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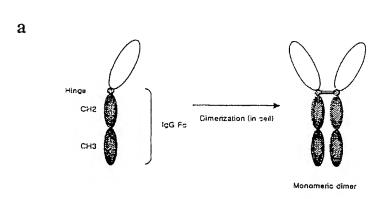
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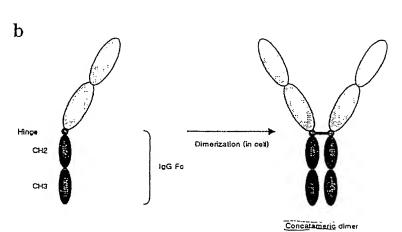
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(54) Title: CONCATAMERIC IMMUNOADHESION





(57) Abstract: Disclosed are concatameric proteins comprising two soluble domains, in which the C-terminus of a soluble domain of a biologically active protein is linked to the N-terminus of an identical soluble domain or a distinct soluble domain of a biologically active protein. Also, the present invention disclosed dimeric proteins formed by formation of intermolecular disulfide bonds at the hinge region of two monomeric proteins formed by linkage of a concatamer of two identical soluble extracellular regions of proteins involving immune response to an Fc fragment of an immunoglobulin molecule, their glycosylated proteins, DNA constructs encoding the monomeric proteins, recombinant expression plasmids containing the DNA constructs, host cells transformed or transfected with the recombinant expression plasmids, and a method of preparing the dimeric proteins by culturing the host cells. Further, the present invention disclosed pharmaceutical or diagnostic compositions comprising the dimeric protein or its glycosylated form.



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CONCATAMERIC IMMUNOADHESION

TECHNICAL FIELD

The present invention relates to concatameric proteins, and more specifically, concatamerized structure of biologically active protein domains where C-terminal end of extracellular soluble domain of biologically active protein is fused to N-terminal end of the same or other extracellular soluble domain of biologically active protein, and dimerization of two concatamers by coupling to hinge region of Fc fragment of immunoglobulin, and glycosylated forms of the concatameric proteins.

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BACKGROUND ART

The activity of cytokine is associated with pathologic severity of inflammatory and /or immune response to various antigenic stimulations. Many antigen specific antibodies and soluble receptors which could recognize cytokines are currently in use to inhibit the function of cytokines for the therapeutic purposes (WO 93/016184, WO 96/02576, WO 96/023067, WO 1997/03682, and US 5,434,131, 5,656,272, 5,977,318, 6,210,661, 6,225,117). Antibodies and soluble receptors inhibit cytokine signal transduction by disturbing interaction between cytokines and their receptors on cell surface.

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Soluble receptors used as functional inhibitors of cytokine that fused to heavy chains of human immunoglobulins were disclosed by Capon et al. (Nature 337:5254, 1989), and thereafter many patents were disclosed inventions related to fusion proteins of soluble receptors and immunoglobulins (US patent 5,521,288, 5,844,095, 6,046,310, 6,090,914, 6,100,383, 6,225,448).

Generally, fusion proteins of soluble receptors and immunoglobulins have following advantages (Capon et al., Nature 337:5254, 1989)

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1. Increase in total avidity to ligand by forming bivalency via dimerization.

2. Increase in blood half-life of proteins, that is, increase in molecular stability

3. Activation of effecter cells by Fc fragment of immunoglobulin heavy chain

4. Convenience of purification by using affinity column, e.g. using protein A Most fusion proteins of receptor extracellular domain and immunoglobulin heavy chain are composed of heavy chain without CH1 domain, which result in dimers not binding to light chains. This structure is more desirable for the function of proteins and receptors involving immune response. For example, TNFR(WO92/16221, WO95/34326)-immunoglobulin fusion proteins disclosed in WO94/06476 and US 5,447,851 have been used for the inhibition of TNF-mediated inflammation. It is well known that TNFR-immunoglobulin fusion proteins have a higher affinity than original monomeric molecules (Lesslauer et al., Eur. J. Immunol. 21:2883, 1991; Ashkenazi et al., Proc. Natl. Acad. Sci. 88:10535, 1991; Peppe et al., J. Exp. Med. 174:1483, 1991; Mohler et al., J. Immunol. 151:1548, 1993).

For the improved inhibition of TNF mediated response, one can increase efficacy by multimerizing soluble extracellular domains of TNFR, CD2, and CTLA-4. For example, when fusion proteins of TNFR's extracellular domains bound with immunoglobulin heavy chain(heavy chain fusion protein) and with light chain(light chain fusion protein) respectively are coexpressed in the same cell, one can produce fusion proteins as a tetrameric form by linking heavy chain to heavy and light chains. This tetramer showed much more increased efficacy than monomeric or dimeric forms as presented by Scallon et al. (Cytokine 7:759, 1995).

However, this method had many difficulties for commercialization such as simultaneous expression of two different fusion genes in the same cell line, remarkably lower production yields of multimeric form; and difficulty in purifying multimeric high

molecular weight forms. For these reasons, immunoglobulin fusion proteins currently in use are only heavy chain fused form.

Therefore, there is considerable demand for the development of methods of producing multimeric protein therapeutics with high yield and efficient purification procedures.

DISCLOSURE OF INVENTION

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The present inventors have manufactured concatameric proteins by fusing the C-terminal end of soluble domain of biologically active protein to the N-terminal end of soluble domain of the same or other biologically active protein by using DNA recombination techniques. Also, the present inventors have dimerized this concatamers by linking it to the hinge region of Fc fragment of immunoglobulin and added more glycosylations by using DNA mutagenesis techniques. And the present inventors have found that concatamerized protein dimers and their glycosylated forms show increased efficacy and stability compared to conventional monomeric fusion proteins.

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Therefore, in one aspect, the present invention provides concatameric proteins where C-terminal end of soluble domain of biologically active proteins is fused to N-terminal end of soluble domain of the same or other biologically active proteins.

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In another aspect, the present invention provides dimeric proteins formed by disulfide bond at hinge region of two monomeric proteins whose concatamerized part is fused to hinge region of Fc fragment of immunoglobulin.

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Also in another aspect, the present invention provides DNA constructs that encode monomeric fusion proteins whose concatamerized domain is fused to hinge region of Fc fragment of immunoglobulins.

Also in another aspect, the present invention provides DNA plasmids comprising a DNA construct that encodes monomeric fusion protein whose concatamerized part is fused to hinge region of Fc fragment of immunoglobulin.

Also in another aspect, the present invention provides host cells transfected or transformed with recombinant DNA plasmids including a DNA construct that encodes monomeric fusion protein whose concatamerized part is fused to hinge region of Fc fragment of immunoglobulin.

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Also in another aspect, the present invention provides a method for culturing the host cells, which were transfected or transformed with recombinant DNA plasmids including a DNA construct that encodes monomeric fusion protein whose concatamerized part is fused to hinge region of Fc fragment of immunoglobulin, under culture condition for expression of DNA constructs encoding concatameric fusion protein coupled to hinge region of Fc fragment of immunoglobulin, and manufacturing dimeric concatamers formed by disulfide bond at hinge region of two monomeric concatamers described as above including the process of purification of the proteins described as above from cell culture.

Also in another aspect, the present invention provides a method for culturing the host cells, which were transfected or transformed with recombinant DNA plasmids including a DNA construct that encodes monomeric fusion protein whose concatamerized part of immunomudulatory function is fused to hinge region of Fc fragment of immunoglobulin and is inserted with glycosylation motifs, under the best condition which is suitable for expression of DNA constructs that encode monomeric fusion protein whose concatamerized part of immune function is fused to hinge region of Fc fragment of immunoglobulin, and for manufacturing glycosylated dimers formed by disulfide bond at hinge region of two monomeric proteins described as above including the process of purification of the glycosylated proteins described as above from cell culture.

Also in another aspect, the present invention provides DNA primers for inserting glycosylation motif into the DNA constructs that encode monomeric fusion

proteins whose concatamerized part is fused to hinge region of Fc fragment of immunoglobulins.

Also in another aspect, the present invention provides the glycosylated dimers formed by disulfide bond at hinge region of two monomeric proteins whose concatamerized part involving immune response is fused to hinge region of Fc fragment of immunoglobulins.

Also in another aspect, the present invention provides the pharmaceutical compositions comprising dimers formed by disulfide bond at hinge region of two monomeric proteins whose concatamerized part involving immune response is fused to hinge region of Fc fragment of immunoglobulins in a pharmaceutically effective amount and in a pharmaceutically acceptable carrier.

Also in another aspect, the present invention provides the pharmaceutical compositions comprising glycosylated dimers formed by disulfide bond at hinge region of two monomeric proteins whose concatamerized part involving immune response is fused to hinge region of Fc fragment of immunoglobulins in a pharmaceutically effective amount and in a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a schematic view showing a process of preparing a DNA construct encoding a conventional simple fusion monomeric protein through polymerase chain reaction (PCR);

Fig. 2 is a schematic view showing a process of preparing a DNA construct encoding a concatameric fusion monomeric protein according to the present invention through PCR;

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Fig. 3a shows structures of [TNFR/Fc]₂, [CD2/Fc]₂ or [CTLA4/Fc]₂ fusion proteins, which are simple fusion dimeric proteins formed through homodimerization in cells of TNFR/Fc, CD2/Fc or CTLA4/Fc fusion proteins as examples of conventional simple fusion monomeric proteins;

Fig. 3b shows structures of [TNFR-TNFR/Fc]₂, [CD2-CD2/Fc]₂ or [CTLA4-CTLA4/Fc]₂ fusion proteins, which are concatameric fusion dimeric proteins formed through homodimerization in cells of TNFR-TNFR/Fc, CD2-CD2/Fc or CTLA4-CTLA4/Fc fusion proteins as embodiments of the concatameric fusion dimeric protein according to the present invention;

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Fig. 4a shows a structure of [TNFR1-TNFR1/Fc]₂, as an embodiment of a concatameric fusion dimeric protein according to the present invention;

Fig. 4b shows a structure of [TNFR2-TNFR2/Fc]₂, as another embodiment of the concatameric fusion dimeric protein according to the present invention;

Fig. 4c shows a structure of [CD2-CD2/Fc]₂, as a further embodiment of the concatameric fusion dimeric protein according to the present invention;

Fig. 4d shows a structure of [CTLA4-CTLA4/Fc]₂, as a still further embodiment of the concatameric fusion dimeric protein according to the present invention;

Fig. 5 is a diagram showing a process of constructing a recombinant expression plasmid pTR11Ig-Top10' expressing a concatameric fusion monomeric protein TNFR1-TNFR1/Fc according to the present invention;

Fig. 6 is a diagram showing a process of constructing a recombinant expression plasmid pCD22Ig expressing a concatameric fusion monomeric protein CD2-CD2/Fc according to the present invention;

Fig. 7 is a map of a recombinant expression plasmid pTR11Ig-Top10' expressing a concatameric fusion monomeric protein TNFR1-TNFR1/Fc according to the present invention;

Fig. 8 is a map of a recombinant expression plasmid pTR22Ig-Top10' expressing a concatameric fusion monomeric protein TNFR1-TNFR1/Fc according to the present invention;

Fig. 9 is a map of a recombinant expression plasmid pCD22Ig expressing a concatameric fusion monomeric protein CD2-CD2/Fc according to the present invention;

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Fig. 10 is a map of a recombinant expression plasmid pCT44Ig expressing a concatameric fusion monomeric protein CTLA4-CTLA4/Fc according to the present invention;

Fig. 11 is a map of a recombinant expression plasmid pTR11Ig-MG expressing a concatameric fusion monomeric protein mgTNFR1-TNFR1/Fc containing four glycosylation motif peptides according to the present invention;

Fig. 12 is a map of a recombinant expression plasmid pTR22Ig-MG expressing a concatameric fusion monomeric protein mgTNFR2-TNFR2/Fc containing two glycosylation motif peptides according to the present invention;

Fig. 13 is a map of a recombinant expression plasmid pCD22Ig-MG expressing a concatameric fusion monomeric protein mgCD2-CD2/Fc containing two glycosylation motif peptides according to the present invention;

Fig. 14 is a map of a recombinant expression plasmid pCT44Ig-MG expressing a concatameric fusion monomeric protein mgCTLA4-CTLA4/Fc containing three glycosylation motif peptides according to the present invention;

Fig. 15 shows a result of SDS-PAGE of purified concatameric fusion dimeric proteins [TNFR1-TNFR1/Fc]₂ and [TNFR2-TNFR2/Fc]₂ under reducing or non-reducing conditions;

Fig. 16 is a graph showing inhibitory effect of the conventional simple fusion dimeric proteins $[TNFR1/Fc]_2(\bullet)$ and $[TNFR2/Fc]_2(\bigcirc)$ and the concatameric fusion dimeric proteins $[TNFR1-RNFR1/Fc]_2(\blacktriangledown)$ and $[TNFR2-TR2Fc]_2(\bigtriangledown)$ according to the present invention against cytotoxic activity of TNF-alpha;

Fig. 17 is a graph showing inhibitory effect of the conventional simple fusion dimeric proteins $[TNFR1/Fc]_2(\bullet)$ and $[TNFR2/Fc]_2(\bigcirc)$ and the concatameric fusion dimeric proteins $[TNFR1-RNFR1/Fc]_2(\blacktriangledown)$ and $[TNFR2-TR2Fc]_2(\bigtriangledown)$ according to the present invention against cytotoxic activity of TNF-beta;

Fig. 18 is a graph showing inhibitory effect of the conventional simple fusion dimeric protein $[CD2/Fc]_2(\bullet)$, the known immunosuppressive agent cyclosporin A (\blacktriangledown) and the concatameric fusion dimeric protein $[CD2-CD2/Fc]_2(\bigcirc)$ according to the present invention on the proliferation of active T lymphocytes;

Fig. 19 is a graph showing inhibitory effect of the conventional simple fusion

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dimeric protein [CTLA4/Fc]₂(●), the known immunosuppressive agent cyclosporin A (▼) and the concatameric fusion dimeric protein [CTLA4- CTLA4/Fc]₂ (O) according to the present invention on the proliferation of active T lymphocytes;

Fig. 20 is a graph showing blood half-life of the conventional simple fusion dimeric protein [TNFR1/Fc]₂(\bullet), the concatameric dimeric protein [TNFR1-TNFR1/Fc]₂ (\bigcirc) and a glycosylated concatameric fusion dimeric protein [mgTNFR1-TNFR1/Fc]₂ (∇) according to the present invention;

Fig. 21 is a graph showing blood half-life of the conventional simple fusion dimeric protein [CD2/Fc]₂(●), the concatameric fusion dimeric protein [CD2-CD2/Fc]₂ (○) and a glycosylated concatameric fusion dimeric protein [mgCD2-CD2/Fc]₂ (▽) according to the present invention;

Fig. 22 is a graph showing blood half-life of the conventional simple fusion dimeric protein [CTLA4/Fc]₂(●), the concatameric fusion dimeric protein [CTLA4-CTLA4/Fc]₂(○) and a glycosylated concatameric fusion dimeric protein [mgCTLA4-CTLA4/Fc]₂(▽) according to the present invention; and

Fig. 23 is a graph showing inhibitory effect of PBS (●) as a control, the conventional simple fusion dimeric proteins [TNFR1/Fc]₂(■) and [TNFR2/Fc]₂(▲), and concatameric fusion dimeric proteins [TNFR1-TNFR1/Fc]₂ (×) and [TNFR2-TNFR2/Fc]₂ (△) according to the present invention on the induction of collagen-induced arthritis (CIA) in DBA/1 mice.

BEST MODE FOR CARRYING OUT THE INVENTION

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The present invention is generally directed to concatameric proteins, and more particularly, to immunoadhesion molecules. Immunoadhesion molecules are typically formed by fusion of the Fc fragment of immunoglobulin (Ig) to a ligand-binding region of a receptor or an adhesion molecule, and thus have a structure similar to that of an antibody. The typical immunoadhesion molecules known in the art have a structure of an antibody in which the variable region is substituted with a ligand-binding region of a receptor while retaining the Fc fragment. A wide variety of immunoadhesion molecules are suggested in the literature. However, immunoadhesion molecules according to the

present invention have different structure with the conventional immunoadhesion molecules, and there is also no prior art predicting or describing preparation of the immunoadhesion molecules according to the present invention.

Definition of Terms

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For full understanding of the characteristic structure of the immunoadhesion molecules according to the present invention, exact definitions of the terms used in the present invention are given as follows. In general, all of the technical and scientific terms being not additionally defined in the present invention have meanings commonly used in the art. However, although having meanings commonly used in the art, the following terms are defined to give a clearer understanding of their meanings and make the scope of the present invention clear, as follows.

The term "immunoglobulin", as used herein, refers to protein molecules being

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produced in B cells and serving as antigen receptors specifically recognizing a wide The molecules have a Y-shaped structure consisting of two identical variety of antigens. light chains (L chains) and two identical heavy chains (H chains), in which the four chains are held together by a number of disulfide bonds, including the disulfide bridge between the H chains at the hinge region. The L and H chains comprise variable and constant The L chain variable region associates with the H chain variable region, thus producing two identical antigen-binding regions. According to features of the constant regions of H chains, immunoglobulins (Ig) are classified into five isotypes, A (IgA), D (IgD), E (IgE), G (IgG) and M (IgM). Each subtype possesses unique structural and biological properties. For example, IgG has slightly different Fc structure, compared In addition, IgG and IgA have a number of subtypes. with other isotypes. example, the human IgG isotype has four subtypes, IgG1, IgG2, IgG3 and IgG4, which have γ1, γ2, γ3 and γ4 H chains, respectively. Biological functions of immunoglobulin molecules, such as complement activation, Fc receptor-mediated phagocytosis and determinants structural mediated by cytotoxicity, are antigen-dependent (complementarity-determining regions) in the Fc region of H chains. Such an Fc region of H chains is used for construction of dimeric proteins according to the present

invention, and may be derived from all isotypes and subtypes of immunoglobulin as described above.

The term "Fc fragment of an immunoglobulin molecule", as used herein, refers to a fragment having no antigen-binding activity and being easily crystallized, which comprises a hinge region and CH2 and CH3 domains, and a portion responsible for binding of an antibody to effector materials and cells. Therefore, the Fc fragment mentioned in the present invention can be different from that described in some literatures, but includes the hinge region. Such description of the Fc fragment is given to supply convenience in describing the present invention, and will be fully understood by those of ordinary skill in the art with reference to the specification of the present invention and the accompanying drawings.

The term "biologically active protein", as used herein, refers to a protein, peptide or polypeptide having generally physiological or pharmaceutical activities, which retains a part of its native activities after forming a concatamer or immunoadhesion molecule. The term "biological activity", as used herein, is not limited in meaning to physiological or pharmaceutical activities. For example, some concatamers, such as those containing an enzyme can catalyze a reaction in an organic solvent. Similarly, some high-molecular weight fusion molecules containing concanavalin A or an immunoglobulin molecule are useful as diagnostic agents in laboratories.

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Non-limiting examples of the protein, peptide or polypeptide include hemoglobin, serum proteins (e.g., blood factors including factor VII, VIII and factor IX), immunoglobulin, cytokines (e.g., interleukin), α -, β - and γ -interferon, colony-stimulating agent (e.g., G-CSF and GM-CSF), platelet-derived growth factor (PDGF), and phospholipase activating proteins (PLAPs). Other typical biological or therapeutic proteins include insulin, plant proteins (e.g., lectin and ricin), tumor necrosis factor (TNF) and its related alleles, growth factors (e.g., tissue growth factors and endothelial growth factors such as TGF α or TGF β), hormones (e.g., follicle-stimulating hormone, thyroid-stimulating hormone, antidiuretic hormone, pigment-concentrating or dispersing hormones and parathyroid hormone, luteinizing hormone-releasing hormone and its derivatives, calcitonin, calcitonin gene related peptide (CGRP), synthetic enkephalin, somatomedin, erythropoietin, hypothalamus releasing factors, prolactin, chronic gonadotrophin, tissue

plasminogen-activating agents, growth hormone-releasing peptide (GHRP), and thymic humoral factor (THF). The immunoglobulins include IgG, IgE, IgM, IgA, IgD and fragments thereof. Some proteins such as interleukin, interferon or colony-stimulating factor may be produced in a non-glycosylated form using DNA recombinant techniques. The non-glycosylated proteins may be useful as biologically active materials in the present invention.

In addition, the biologically active materials useful in the present invention include any polypeptide, which has bioactivity in vivo. Examples of the biologically active materials include peptides or polypeptides, fragments of an antibody, single chain-binding proteins (see U.S. Pat. No. 4,946,778), binding molecules including fusion polypeptides of antibodies or their fragments, polyclonal antibodies, monoclonal antibodies, and catalytic antibodies. Other examples of the biologically active materials include allergen proteins, such as ragweed, antigen E, honeybee venom, or allergen of mites.

In addition, the biologically active material useful in the present invention includes enzymes. Examples of the enzymes include carbohydrate-specific enzymes, proteinases, oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. In detail, non-limiting examples of the enzymes include asparaginase, arginase, arginine deaminase, adenosine deaminase, peroxide dismutase, endotoxinase, catalase, chymotrypsin, lipase, uricase, adenosine dephosphatase, tyrosinase, and bilirubin oxidase. Examples of the carbohydrate-specific enzymes include glucose oxidase, glucodase, galactosidase, glucocerebrosidase, and glucouronidase.

The term "proteins involving immune response", as used herein, refers to all proteins mediating cell-to-cell signal transduction during cellular or humoral immune response and thus activating or suppressing immune response. Immunity is a process of protecting "self" from "non-self" such as bacteria or viruses. Immune response is largely divided into cellular and humoral immune response, where T and B lymphocytes play the most important role. T cells, mainly mediating cellular immune response, directly attack and kill virus-infected cells or tumor cells, or help other immune cells by secreting cytokines functioning to induce or activate immune response or inflammation. B cells produce antibodies against non-self foreign materials (antigens) that enter a body,

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such as bacteria or viruses, and such immune response is called cellular immune response. Cell-to-cell signal transduction is an essential process in both cellular and humoral immune responses, in which a signal molecule, that is, a ligand, interacts with a cell surface receptor acting to transduce a specific signal into a cell.

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Representative examples of the proteins involving the immune response according to the present invention include cytokines, cytokine receptors, adhesion molecules, tumor necrosis factor receptor (TNFR), enzymes, receptor tyrosine kinases, chemokine receptors, other cell surface proteins, and soluble ligands. Non-limiting examples of the cytokines include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-17, TNF, TGF, IFN, GM-CSF, G-CSF, EPO, TPO, and M-CSF. Examples of the cytokine receptors, but are not limited to, include growth hormone receptors (GHRs), IL-13R, IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-9R, IL-15R, TNFR, TGFR, IFNR (e.g., IFN-γ R α-chain and IFN-γ R β-chain), interferon-α R, -β R and -γ R, GM-CSFR, G-CSFR, EPOR, cMpl, gp130, and Fas (Apo 1). Non-limiting examples of the enzymes include influenza C hemaglutinin esterase and urokinase. The chemokine receptors are exemplified by CCR1 and CXCR1-4. Examples of the receptor tyrosine kinases, but are not limited to, include TrkA, TrkB, TrkC, Htk, REK7, Rse/Tyro-3, hepatocyte growth factor R, platelet-derived growth factor R, and Flt-1. Examples of other cell surface proteins includes CD2, CD4, CD5, CD6, CD22, CD27, CD28, CD30, CD31, CD40, CD44, CD100, CD137, CD150, LAG-3, B7, B61, \(\beta\)-neurexin, CTLA-4, ICOS, ICAM-1, complement R-2 (CD21), IgER, lysosomal membrane gp-1, α2microglobulin receptor-related proteins, and sodium-releasing peptide R. Non-limiting examples of the soluble ligands include IL-10, heregulin, and keratinocyte growth factors.

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Ligands for the proteins involving immune response according to the present invention and use thereof are well known to those of ordinary skill in the art, as summarized in Tables 1 to 7, below.

TABLE 1
Proteins involving immune response: Adhesion molecules

Adhesion	Ligands	Uses
molecules		Inhibition of in vivo HIV infection; and identification of CD4
CD4	HIV gp120	1
		ting of nontrophile-mediated ling damage; determination of
L-Selectin	GlyCAM-1, CD34	in themas of a ligand by histochemical statume, and
L-Selectin	GIJ GI ZII Z,	i taking and cloping of ligands and determination of their proportion
	Sialyl Lewis ^X	Prevention of neutrophile-mediated lung damage; and determination
E-Selectin	Staryi Lewis	of thermodynamic properties in ligand-binding Prevention of neutrophile-mediated lung damage; and study of
	. X	functions of individual of amino acid residues in binding to cell
P-Selectin	Sialyl Lewis ^x	
		Phagocytosis of erythrocytes in malaria; inhibition of infection with
ICAM-1	CD11a/CD18	-binordings; and anti-inflammation in diabetes
ICAM-2	CD11a/CD18	Study of activation of T cells mediated by T cell receptor
ICAM-3	CD11a/CD18	The tigotion of recentor domains binding to a ligatu
VCAM-1	VLA-4	Study of role of VLA-4 in T lymphocyte migration to dermal
VOI 2		inflammation sites
LFA-3	CD2	Study of role of CD2 in costimulation of T cells Stimulation of nerve reproduction after repair; and functional
L1	Fibroblast growth	Stimulation of nerve reproduction after repair,
glycoprotein	factor receptor	comparison with FGF

TABLE 2
Proteins involving immune response: Enzymes

Enzymes Ligands Influenza C hemaglutinin esterase sialic acid Urokinase Urokinase receptor	Uses Inactive enzyme used in study of tissue-specific expression of ligands Inactive enzyme developed to inhibit cancer metastasis by disturbing urokinase activation
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TABLE 3
Proteins involving immune response: Cytokine receptors

	Liganda	Uses
Cytokine	Ligands	
receptors		Inhibition of IFN-mediated autoimmunity
IFN-γ R α-chain	IFN-γ	Study of structure of subunits of a ligand-receptor complex
IFN-γ R β-chain	IFN-γ	Study of structure of subunits of a figure-receptor company
IL1R	IL-1	Inhibition of IL-1-mediated inflammation
IL4R	Π,-4	Identification of receptor domains participating in ligand
II.AV	10.	binding
= 11	Erythropoietin	Map design of epitopes of anti-ligand antibodies
Erythropoietin R		Isolation and cloning of ligands
cMp1	Thrombopoietin	Study of structure of subunits of a ligand-receptor complex
gp130	IL-6-IL6R	Study of structure of should so a ngara 200-person
<i>5</i> 1	complex	

TABLE 4

Proteins involving immune response: Tumor necrosis factor receptors

		TToo
TNF receptors	Ligands	Uses
TNF R-1	TNF,	Treatment of septic shock, rheumatoid arthritis and other inflammatory
	lymphotoxin-α	diseases; and identification of domains participating in ligand binding
TNF R-2	TNF,	Inhibition of TNF-enriched HIV replication; and prevention of
1141 162	lymphotoxin-α	collagen-induced arthritis in mice
Lymphotoxin-	Lymphotoxin-β	Study of structure of subunits of cell surface lymphotoxin-β
βR	Lymphotoxin p	
	E/A	Treatment of excessive apoptosis and related diseases (e.g., AIDS);
Fas/Apo-	Fas/Apo-	and resistance to apoptosis of lymphocytes and peripheral immune
1/CD95	1/CD95 ligand	and resistance to apoptosis of symphocytes and perspectation and
		tolerance; roles of Fas ligand in T cell-mediated cytotoxicity; and
		isolation and cloning of ligands
CD27	CD27 ligand	Isolation and cloning of ligands
CD30	CD30 ligand	Isolation and cloning of ligands
CD40	gp39	Isolation and cloning of ligands
4-1BB	4-1BB ligand	Identification of tissues containing ligands by histochemical staining,
		isolation and cloning of ligands; and Study of structural determinant of
		potential ligand
OX40	gp34	Isolation and cloning of ligands

TABLE 5
Proteins involving immune response: Receptor tyrosine kinases

Receptor tyrosine	Ligands	Uses
kinases		
TrkA, B, C	Neutropin	Determination of properties of neutropin binding
Htk	Htk ligand	Isolation and cloning of ligands
REK7	AL-1	Isolation and cloning of ligands
Rse/Tyro-3	Protein S, Gas6	Identification of ligands and determination of
RSC/1910 3	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	their properties
Hepatocyte growth	Hepatocyte growth factor	Identification of receptor domains participating in
factor R	Tropatooy to Brown and and	ligand binding
Platelet-derived	Platelet-derived growth	Identification of receptor domains participating in
growth factor R	factor	ligand binding
Flt-1	Vesicular endothelial growth	Determination of properties of ligand binding of
FIL-1	factor (VEGF)	receptors
77.	VEGF	Evaluation of selectivity of receptors for VEGF
Flk-1/KDR	VEGF	versus placenta growth factor

TABLE 6
Proteins involving immune response: Other cell surface proteins

		Uses
Other cell surface	Ligands	USES
proteins	CD20	Study of T cell stimulation by B cells
B7	CD28	Roles of Eck in inflammation
B61	Eck	
β-neurexin	β-neurexin ligand	Determination of properties of a signal sequence from β-neurexin
CD2	LFA-3, CD48	Identification of ligands
CD5	CD5 ligand	Study of T cell stimulation by B cells
CD6	ALCAM	Study of binding activities of cloned ligands
CD22	CD45, other	Identification of ligands; study on roles of CD22 in T-B-
	sialoglycoproteins	cell interaction; and determination of properties of binding
		determinants of sialo-oligo sugar ligands
CD28	B7, B7-2	Study of T cell stimulation by B cells
CD31	CD31	Identification of CD31 domains related to homotype
		binding
CD44	Hyaluronate	Screening of tissues containing ligands by histochemical
	•	staining; and determination of properties of structural
		determinants of ligands
Complement R-2	C3 fragment	Inhibition of reactivity of antibody to immunosuppressive
(CD21)		and cancer therapeutic agents
CTLA-4	B7	Identification of CTLA-4 as a secondary receptor of B7
IgE R	IgE	Inhibition of mast cell-binding of IgE as therapy of allergic
-3		diseases
Lisosome membrane	LAMP-1 ligand	Design of epitope maps of anti-ligand antibodies
gp-1		
α2-microglobulin	gp330	Determination of position of ligands in tissues by
receptor-bound		histochemical staining
proteins		
Sodium-releasing	Sodium-releasing	Design of epitope maps of anti-ligand antibodies; and
peptide R	peptide	preparation of recombinant receptors for structural study

TABLE 7
Proteins involving immune response: Soluble ligands

Soluble ligands	Ligands	Uses
IL-2	IL-2R	Extension of half-life of IL-2 in the circulation system
IL-10	IL-10R	Therapy of septic shock and transplantation rejection; and extension of half-life of IL-10 in the circulation system
Heregulin	Her4/p180 ^{erbB4}	Study of signal transduction by Her4
Keratinocyte growth factor	Keratinocyte growth factor R	Determination of position of receptors by histochemical staining

The term "soluble extracellular domain", as used herein, refers to a portion exposed to the extracellular region of an integral membrane protein penetrating the cell membrane comprising phospholipid, wherein the integral membrane protein contains one or more transmembrane domain made up predominantly of hydrophobic amino acids.

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Such an extracellular domain mainly comprises hydrophilic amino acids, which are typically positioned at the surface of a folded structure of a protein, and thus is soluble in an aqueous environment. For most cell surface receptor proteins, extracellular domains serve to bind specific ligands, while intracellular domains play an important role in signal transduction.

The term "concatamer-linked", as used herein, refers to a state in which two soluble domains of biologically active proteins are linked and thus form a long polypeptide.

The term "concatameric protein", as used herein, means a concatamer-linked protein. For example, the N-terminus of a soluble extracellular domain of a protein involving immune response is linked to the C-terminus of an identical soluble extracellular domain of the protein involving immune response, wherein the C-terminus of the former soluble extracellular domain is linked to the hinge region of an Fc fragment of an immunoglobulin molecule. Thus, two identical soluble extracellular domains of a protein involving immune response form a long polypeptide.

The term "simple fusion monomeric protein", as used herein, refers to a fusion protein having a monomeric structure consisting of a single polypeptide formed by linkage of a soluble extracellular domain of a protein involving immune response to the hinge region of an Fc fragment of an immunoglobulin molecule. A simple fusion monomeric protein may be designated "protein name/Fc" for convenience in the present invention. For example, a simple fusion monomeric protein produced by linkage of an soluble extracellular domain of TNFR1 protein involving immune response to an Fc fragment of an immunoglobulin molecule is designated TNFR1/Fc. If desired, the origin of the Fc fragment may be also specified in the designation. For example, in the case that the Fc fragment is derived from IgG1, the monomeric protein is called TNFR1/IgG1Fc.

The term "simple fusion dimeric protein", as used herein, refers to a fusion protein having a dimeric structure, in which two simple fusion monomeric proteins are joined by formation of intermolecular disulfide bonds at the hinge region. Such a simple fusion dimeric protein may be designated "[protein name/Fc]₂" for convenience in the present invention. For example, when fused by formation of intermolecular disulfide

bonds at the hinge region of two simple fusion monomeric proteins produced by linkage of an soluble extracellular domain of TNFR1 protein and an Fc fragment of an immunoglobulin molecule, the resulting fusion protein having dimeric structure is designated [TNFR1/Fc]₂. In addition, the origin of the Fc fragment may be specified in the designation, if desired. For example, in the case that the Fc fragment is derived from IgG1, the dimeric protein is designated [TNFR1/IgG1Fc]₂.

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The term "concatameric fusion monomeric protein", as used herein, refers to a fusion protein having a monomeric structure consisting of a single polypeptide, in which the N-terminus of a soluble extracellular domain of a protein involving immune response is linked to the C-terminus of an identical soluble extracellular domain of the protein involving immune response, wherein the C-terminus of the former soluble extracellular domain is linked to the hinge region of an Fc fragment of an immunoglobulin molecule. A concatameric fusion monomeric protein may be designated "protein name-protein name/Fc" for convenience in the present invention. For example, when an soluble extracellular domain of TNFR1 of a simple fusion monomeric protein, produced by linkage of the soluble extracellular domain of TNFR1 protein involving immune response and an Fc fragment of an immunoglobulin molecule, is linked to an identical soluble extracellular domain of TNFR1, the resulting concatameric fusion monomeric protein is designated TNFR1-TNFR1/Fc. If desired, the origin of the Fc fragment may be specified in the designation. For example, in the case that the Fc fragment is derived from IgG1, the monomeric protein is designated TNFR1-TNFR1/IgG1Fc.

The term "concatameric fusion dimeric protein", as used herein, refers to a fusion protein having a dimeric structure, in which two concatameric fusion monomeric proteins are fused by formation of intermolecular disulfide bonds at the hinge region. A concatameric fusion dimeric protein may be designated "[protein name-protein name/Fc]₂" for convenience in the present invention. For example, when two concatameric fusion monomeric proteins, each of which is produced by linkage of a TNFR1 soluble extracellular domain of a simple fusion monomeric protein to an identical soluble extracellular domain of TNFR1 protein involving immune response, are fused by formation of intermolecular disulfide bonds at the hinge region, the resulting fusion protein having dimeric structure is designated [TNFR1-TNFR1/Fc]₂, wherein the simple

fusion monomeric protein is formed by linkage of the TNFR1 soluble extracellular domain to an Fc fragment from an immunoglobulin molecule. If desired, the origin of the Fc fragment may be specified in the designation. For example, in the case that the Fc fragment is derived from IgG1, the fusion protein is designated [TNFR1-TNFR1/IgG1Fc]₂.

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The term "vector", as used herein, means a DNA molecule serving as a vehicle capable of stably carrying exogeneous genes into host cells. For useful application, a vector should be able to replicate, have a system for introducing itself into a host cell, and possess selectable markers. The exogeneous genes, for example, include, a DNA construct encoding a concatameric fusion monomeric protein.

The term "recombinant expression plasmid", as used herein, refers to a circular DNA molecule carrying exogeneous genes operably linked thereto to be expressed in a host cell. When introduced into a host cell, the recombinant expression plasmid has the ability to replicate regardless of host chromosomal DNA, copy itself at a high copy number, and to produce heterogeneous DNA. As generally known in the art, in order to increase the expression level of a transfected gene in a host cell, the gene should be operably linked to transcription and translation regulatory sequences functional in a host cell selected as an expression system. Preferably, the expression regulation sequences and the exogeneous genes may be carried in a single expression vector containing bacteria-selectable markers and a replication origin. In case that eukaryotic cells are used as an expression system, the expression vector should further comprise expression markers useful in the eukaryotic host cells.

The term "operably linked", as used herein, means an arrangement of elements of a vector, in which each element is capable of performing its innate function. Therefore, a control sequence operably linked to a coding sequence can influence expression of the coding sequence. A control sequence acting to induce expression of a coding sequence does not have to be adjacent to the coding sequence. For example, when an intervening sequence is present between a promoter sequence and a coding sequence, the promoter sequence may still be "operably linked" to the coding sequence.

Host cells used in the present invention may be prokaryotic or eukaryotic. In addition, host cells having high introduction efficiency of foreign DNA and having high

expression levels of an introduced gene may be typically used. Examples of the host cells useful in the present invention include prokaryotic and eukaryotic cells such as *E. coli, Pseudomonas* sp., *Bacillus* sp., *Streptomyces* sp., fungi or yeast, insect cells such as *Spodoptera frugiperda* (Sf9), animal cells such as Chinese hamster ovary cells (CHO) or mouse cells, African green monkey cells such as COS 1, COS 7, human embryonic kidney cells, BSC 1, BSC 40 or BMT 10, and tissue-cultured human cells. When cloning a DNA construct encoding the fusion protein according to the present invention, host cells are preferably animal cells. When using COS cells, since SV40 large T antigen is expressed in COS cells, a plasmid carrying a SV 40 replication origin may be present as a multicopy episome and thus allows high expression of an exogeneous gene. A DNA sequence introduced into a host cell may be homogeneous or heterogeneous to the host cell, or a hybrid DNA sequence containing a homogenous or heterogeneous DNA sequence.

In order to express a DNA sequence encoding the concatameric fusion protein according to the present invention, a wide variety of combinations of host cells as an expression system and vectors may be used. Expression vectors useful for transforming eukaryotic host cells contain expression regulation sequences from, for example, SV40, bovine papillomavirus, adenovirus, adeno-associated viruses, cytomegalovirus and retroviruses. Expression vectors useful in bacterial host cells include bacterial plasmids from $E.\ coli$, which are exemplified by pBluescript, pGEX2T, pUC, pCR1, pBR322, pMB9 and derivatives thereof, plasmids having a broad range of host cells, such as RP4, phage DNAs, exemplified by a wide variety of λ phage derivatives including λ gt10, λ gt11 and NM989, and other DNA phages, exemplified by filamentous single-stranded DNA phages such as M13. Expression vectors useful in yeast cells include 2μ plasmid and derivatives thereof. Expression vectors useful in insect cells include pVL 941.

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The term "transformation", as used herein, means introducing DNA into a suitable host cell so that the DNA is replicable, either as an extrachromosomal element, or by chromosomal integration.

The term "transfection", as used herein, refers to the taking up of an expression vector by a suitable host cell, whether any coding sequences are in fact expressed or not.

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The term "signal sequence", as used herein, means an amino acid sequence mediating transport of an expressed protein to the outside of the cell membrane, and is also

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called a "leader sequence". Cell surface proteins or secretory proteins, which are transported to the outside of the cell membrane, have an N-terminal sequence typically cut by signal peptidase in the cell membrane. Such a N-terminal sequence is called a signal sequence or signal peptide, or a leader sequence or leader peptide. Secretory (or transported) proteins or all proteins present outside of the cell membrane or in the extracellular environment have a specific signal sequence. There is no specific homology between such signal sequences and same proteins have different signal sequences according to their origin. Secondary structure or distribution of nonpolar and charged residues is more important for proper function of the signal sequences than primary structures thereof. Although not having specific homology, the signal sequences share several common features, as follows. The signal sequences contain an N domain at their N-termini, which is a hydrophilic region comprising one or more positively charged residues, and an Hdomain follows the N domain, which is a somewhat long hydrophobic region. In the case The N domain of E. coli, the signal sequence comprises about 18-30 amino acids. contains many cationic amino acids such as Lys or Arg, and thus has a net positive charge. Many hydrophobic amino acids such as Ala or Leu are found in the H domain, and polar or charged amino acids such as Pro, Lys, Arg, Asn or Glu are rarely in the H domain. A large number of amino acids such as Ala and Leu residues form an α-helical structure to facilitate membrane penetration. A C domain is positioned between the H domain and an actually secreted portion of a protein. The C domain is less hydrophobic, and contains a sequence capable of being recognized by signal peptidase such as LebB or LspA. have been no reports about an exact site cleaved by the signal peptidase, but the signal peptidase is typically known to mostly cleave behind the Ala-X-Ala sequence in the C domain. Preproteins containing the above-mentioned signal sequence arrive at the cell membrane through interaction with several proteins, and fold to their mature forms through cleavage of a specific region of a signal peptide. Such a signal sequence is very important in strategies to express a desired protein on the cell surface or in the extracellular environment. Foreign proteins and fusion proteins should be stably transported to the extracellular environment at high efficiency. Typically, cell surface proteins having excellent secretory ability are useful for cell surface expression of foreign proteins or fusion

proteins, which typically have secretory signal sequences capable of offering excellent secretion efficiency.

<u>Preparation of the concatameric fusion dimeric protein according to the present invention</u>

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The concatameric fusion dimeric protein according to the present invention is generally prepared by (a) preparing a DNA construct encoding a simple fusion monomeric protein using a gene encoding an Fc fragment of an immunoglobulin molecule and a gene encoding a soluble extracellular domain of a protein involving immune response; (b) inserting by polymerase chain reaction (PCR) a recognition sequence of a restriction enzyme into the prepared simple fusion monomeric protein-encoding DNA construct and an identical gene to the gene encoding a soluble extracellular domain of a protein involving immune response, respectively; (c) cleaving the recognition sequence of a restriction enzyme in the simple fusion monomeric protein-coding DNA construct and the gene encoding a soluble extracellular domain of a protein involving immune response using the restriction enzyme recognizing the recognition sequence; (d) ligating the cleaved DNA fragments using ligase to produce a DNA construct encoding a concatameric fusion monomeric protein (see, Fig. 2); (e) operably linking the prepared DNA construct encoding a concatameric fusion monomeric protein to a vector to produce a recombinant expression plasmid; (f) transforming or transfecting a host cell with the recombinant expression plasmid; and (g) culturing the transformant or transfectant under conditions suitable for expression of the DNA construct encoding a concatameric fusion monomeric protein and then isolating and purifying a concatameric fusion dimeric protein of interest.

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A DNA fragment encoding a soluble extracellular domain of a protein involving immune response is produced by PCR using a primer containing a recognition sequence of a specific restriction enzyme and a sequence encoding a leader sequence, and a primer containing an antisense sequence encoding the 3' end of the soluble extracellular domain and a portion of the 5' end of a specific region of Fc fragment of an immunoglobulin molecule.

A DNA fragment encoding a specific region of the Fc fragment of an immunoglobulin molecule is produced by PCR using a primer having a sequence encoding a portion of the 3' end of the soluble extracellular domain of the protein involving immune response and a sequence encoding the 5' end of the specific region of the Fc fragment of an immunoglobulin molecule, and another primer having an antisense sequence encoding a recognition sequence of a specific restriction enzyme and the 3' end of a specific region of the Fc fragment of an immunoglobulin molecule.

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The DNA fragment encoding a soluble extracellular domain of a protein involving the immune response and the DNA fragment encoding a specific region of Fc fragment of an immunoglobulin molecule, as described above, are mixed in a test tube. After denaturation, the DNA is re-annealed. Then, a complete double-stranded DNA fragment is produced by polymerization using DNA polymerase at the 3' end of each DNA hybrid. Using the resulting double-stranded DNA fragment, another polymerase chain reaction (PCR) is carried out with the primer having a sequence encoding a soluble extracellular domain of a protein involving immune response and the primer encoding the 3' end of a specific region of the Fc fragment of an immunoglobulin molecule, in order to amplify a immunoglobulin fusion gene comprising a sequence corresponding to the DNA fragment encoding a soluble extracellular domain of a protein involving immune response and a sequence corresponding to the DNA fragment encoding a specific region of the Fc fragment of an immunoglobulin molecule.

An recognition sequence of a restriction enzyme is introduced by PCR into the amplified immunoglobulin fusion gene and the DNA fragment having a sequence encoding a soluble extracellular domain of a protein involving the immune response. The recognition sequence is then cleaved with the restriction enzyme and the cleaved regions are ligated using ligase, thus producing a concatameric immunoglobulin fusion gene.

The immunoglobulin fusion gene may further include a signal sequence to stimulate extracellular secretion of a protein encoded thereby. For example, the CTLA-4 molecule contains a unique leader sequence having highly hydrophilic redundancy at its N-terminus, and which is abnormally long and highly water-soluble (Harper, K. et al., J. Immunol. 147:1037-1044; and Brunet, J.F. Nature 328:267-270, 1987). Generally, most

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cell surface proteins or secretory proteins have a leader sequence comprising 20-24 highly hydrophobic amino acids at their N-termini. However, the CTLA-4 molecule used in the present invention comprises a total of 37 residues: 16 hydrophilic amino acids at its N-terminus, and 21 highly hydrophobic amino acids typical in its transmembrane regions. In the conventional method of preparing CTLA4Ig fusion proteins, the leader sequence of the CTLA-4 molecule was substituted with a leader sequence of oncostatin M (Linsley, P.S. et al., J. Exp. Med. 174:561-569, 1991) or IL-6 (Yamada, A, et al., Microbiol. Immunol. 40:513-518, 1996). The present inventors demonstrated that a CTLA-4 molecule containing a leader sequence having a "MRTWPCTLLFFIPVFCKA" sequence consisting of 16 sequence acid amino of the instead "ACLGFQRHKAQKNLAA", is preferable, and the secretion of an expressed protein to the extracellular environment is easily achieved, as disclosed in International Pat. Publication No. WO98/31820.

A recombinant expression plasmid is prepared by inserting the immunoglobulin fusion gene into a vector, and then introduced to a host cell to produce a transformant or transfectant. A concatameric fusion dimeric protein of interest may be obtained by culturing the transformant or transfectant cell and isolating and purifying a concatameric fusion protein.

A host cell useful for preparation of the concatameric fusion dimeric protein according to the present invention is preferably selected from among bone marrow cell lines, CHO cells, monkey COS cells, human embryonic kidney 293 cells, and baculovirus-infected insect cells. A polypeptide of interest, produced in such an expression system, is secreted to culture medium as an inclusion body. Then, the concatameric fusion dimeric protein can be purified by affinity chromatography using a protein A or protein G column. In fact, effective mammalian expression systems and such purification systems are very useful in expressing proteins involving immune response in a dimeric form, and isolation of such proteins.

Preparation of the glycosylated concatameric fusion dimeric protein according to the present invention

Secretory proteins produced in eukaryotic cells as host cells are modified by glycosylation. Glycosylation is known to influence in vivo stability and functionality as well as physical properties of a protein. Therefore, a preferred aspect of the present invention includes facilitating production of a concatameric fusion dimeric protein of interest using recombinant DNA techniques and the above-mentioned animal cell lines as host cells, and linking additional sugar chains to a soluble extracellular domain of a protein involving immune response.

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Two glycosylation patterns are known. One is O-linked glycosylation, in which an oligosaccharide is linked to a serine or threonine residue, and the other is N-linked glycosylation, in which an oligosaccharide is linked to asparagine residue. N-linked glycosylation occurs at a specific amino acid sequence, particularly, Asn-X-Ser/Thr, wherein X is any amino acid excluding proline. N-linked oligosaccharide has a structure distinct from O-linked oligosaccharide, and glycosylated residues found in the N-linked type also differ from the O-linked type. For example, N-acetylgalactosamine is invariably linked to serine or threonine in O-linked oligosaccharide, while N-acetylglucosamine is linked to asparagines in all of N-linked oligosaccharides. The O-linked oligosaccharides generally contain only 1-4 sugar residues. In contrast, the N-linked oligosaccharides comprise 5 or more sugar residues, essentially including N-acetylglucosamine and mannose.

In accordance with the present invention, to allow additional O-linked or N-linked glycosylation, one or more nucleotides in a DNA sequence encoding a soluble extracellular domain of a protein involving immune response are altered, and the resulting DNA is expressed in a suitable animal host cell to induce glycosylation using the host system. In accordance with an aspect of the present invention, the glycosylated concatameric fusion dimeric protein according to the present invention may be prepared by altering a DNA sequence encoding a soluble extracellular domain of a protein involving immune response to induce or increase N-linked glycosylation by adding the sequence Asn-X-Ser/Thr.

Alteration of a DNA sequence to introduce glycosylation may be performed according to the conventional method common in the art. In a preferred aspect of the present invention, to protect the concatameric fusion protein, especially the two soluble

extracellular domains, from attack of intercellular proteinases and thus increase its halflife in serum, a DNA construct encoding a multiglycosylated concatameric fusion monomeric protein may be prepared using PCR, which introduces multiglycosylation of the present invention, glycosylation motif peptide sequences may be introduced into the concatameric fusion protein, as follows. A DNA fragment is prepared by performing PCR using a primer encoding a leader sequence of a soluble extracellular domain and EcoRI restriction site, and an antisense primer in which a portion of a nucleotide sequence encoding a portion of the 3' end of a first soluble extracellular domain and a portion of the 5' end of a second soluble extracellular domain is substituted with glycosylation motif Another DNA fragment is prepared by performing PCR using a primer in which a portion of a nucleotide sequence encoding a portion of the 3' end of a first soluble extracellular domain and a portion of the 5' end of a second soluble extracellular domain is substituted with glycosylation motif sequences, and an antisense primer encoding the 3' end of Fc portion of IgG1 and XbaI restriction site. Then, secondary PCR is carried out in a test tube using the two DNA fragments.

In accordance with an embodiment of the present invention, the soluble extracellular domains useful in the present invention include soluble extracellular domains of TNFR1, TNFR2, CD2 and CTLA-4. Their application will be described in detail with reference to accompanying figures, sequence listing and examples.

Tumor necrosis factor-alpha (TNF-α), which is known as the hormone cachectin, and tumor necrosis factor-beta (TNF-β), which is also known as lymphotoxin, are multifunctional cytokines, inducing inflammation, cellular immune response, septicemia, cytotoxicity, cachexia, rheumatoid arthritis, inflammation-related diseases (Tartaglia, L.A. et al., Immunol. Today 13:151,1992), and antiviral reaction (Butler, P., Peptide Growth Factor II, 1990, Springer-Verlag, Berlin, pp.39-70). Such actions of TNF-α and TNF-β, including cytotoxic activity, originate from their binding to TNF receptors in a trimeric form (Eck, M.J. et al., J. Biol. Chem. 267:2119, 1992). As TNF receptors, 55 kDa-type I (TNFR1 or p55) and about 75 kDa-type II (TNFR2 or p75) are known (Smith, C.A. et al., Science 248:1019, 1990; Loetscher, H. et al., Cell 61:351, 1990; and Schall et al., Cell 61:361, 1990). The two receptors have similar affinity for TNF-α and TNF-β (Schall et

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al., Cell 61:361, 1990). Immunoglobulin fusion proteins of such soluble receptors have effects of inhibiting the action of TNF- α and TNF- β by inhibiting binding of TNF- α and TNF- β to their receptors on the cell surface, which is known to be effective in reducing TNF-dependent inflammation.

Among cell surface antigens regulating immune response, the costimulatory molecule CD2 and CTLA-4, inducing secondary stimulation to give sufficient activation of T cells, when being in a soluble form, also can be used for therapy of diverse immunological diseases according to the same method as TNF receptors. Immune response is accomplished by binding of cell surface antigen molecules of antigen presenting cells (APC) to specific receptors of T lymphocytes, that is, T lymphocytes and leukocyte-function-antigen molecules of APC, and when a costimulatory signal as a secondary signal is not produced during antigen-presenting, T lymphocytes are removed by apoptosis or inhibition of clonal activation. CD2 is a leukocyte-function-antigen on T lymphocytes, binding to LFA-3 on APC, and participates in adhesion and costimulation of leukocytes, as well as stimulating T cell activation through costimulation with CD28. CTLA-4 is expressed after activation of T lymphocytes, and its expression level is increased in the resting phase. CTLA-4 has a binding affinity to the B7 molecule of APC over 20 times higher than that of CD28, and transduces signals inhibiting T lymphocyte activation after binding to B7.

In a specific aspect of the present invention, there are provided a concatameric fusion monomeric protein TNFR1-TNFR1/Fc, designated by SEQ ID NO: 6; a concatameric fusion monomeric protein TNFR2-TNFR2/Fc, designated by SEQ ID NO: 8; a concatameric fusion monomeric protein CD2-CD2/Fc, designated by SEQ ID NO: 18; and a concatameric fusion monomeric protein CTLA4-CTLA4/Fc, designated by SEQ ID NO: 20.

In another specific aspect of the present invention, there are provided a DNA construct (TNFR1-TNFR1-IgG) encoding a concatameric fusion monomeric protein TNFR1-TNFR1/Fc, designated by SEQ ID NO: 5; a DNA construct (TNFR2-TNFR2-IgG) encoding a concatameric fusion monomeric protein TNFR2-TNFR2/Fc, designated by SEQ ID NO: 7; a DNA construct (CD2-CD2-IgG) encoding a concatameric fusion monomeric protein CD2-CD2/Fc, designated by SEQ ID NO: 17; and a DNA construct

(CTLA4-CTLA4-IgG) encoding a concatameric fusion monomeric protein CTLA4-CTLA4/Fc, designated by SEQ ID NO: 19.

In a further specific aspect of the present invention, there are provided a recombinant expression plasmid pTR11Ig-Top10' operably linked to a DNA construct encoding a concatameric fusion monomeric protein TNFR1-TNFR1/Fc, designated by SEQ ID NO: 5; a recombinant expression plasmid pTR22Ig-Top10' operably linked to a DNA construct encoding a concatameric fusion monomeric protein TNFR2-TNFR2/Fc, designated by SEQ ID NO: 7; a recombinant expression plasmid pCD22Ig operably linked to a DNA construct encoding a concatameric fusion monomeric protein CD2-CD2/Fc, designated by SEQ ID NO: 17; and a recombinant expression plasmid pCT44Ig operably linked to a DNA construct encoding a concatameric fusion monomeric protein CTLA4-CTLA4/Fc, designated by SEQ ID NO: 19. The recombination expression plasmids are deposited in Korean Culture Center of Microorganisms (KCCM) and are assigned accession Nos. KCCM-10288, KCCM-10291, KCCM-10402 and KCCM-10400, The KCCM deposit will be maintained under the terms of the Budapest respectively. Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

In a further specific aspect of the present invention, there are provided a mammalian host cell (e.g., TR11Ig-CHO) transformed or transfected with a recombinant expression plasmid pTR11Ig-Top10' operably linked to a DNA construct encoding a concatameric fusion monomeric protein TNFR1-TNFR1/Fc, designated by SEQ ID NO: 5; a mammalian host cell (e.g., TR22Ig-CHO) transformed or transfected with a recombinant expression plasmid pTR22Ig-Top10' operably linked to a DNA construct encoding a concatameric fusion monomeric protein TNFR2-TNFR2/Fc, designated by SEQ ID NO: 7; a mammalian host cell transformed or transfected with a recombinant expression plasmid pCD22Ig operably linked to a DNA construct encoding a concatameric fusion monomeric protein CD2-CD2/Fc, designated by SEQ ID NO: 17; and a mammalian host cell transformed or transfected with a recombinant expression plasmid pCT44Ig operably linked to a DNA construct encoding a concatameric fusion monomeric protein CTLA4-CTLA4/Fc, designated by SEQ ID NO: 19. Chinese hamster ovary cell line TR11Ig-CHO transfected with the recombinant expression

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plasmid pTR11Ig-Top10' and Chinese hamster ovary cell line TR22Ig-CHO transfected with the recombinant expression plasmid pTR22Ig-Top10' are deposited in KCCM and are assigned accession Nos. KCLRF-BP-00046 and KCLRF-BP-00049, respectively. The KCCM deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

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In a still further specific aspect of the present invention, there are provided a concatameric fusion monomeric protein mgTNFR1-TNFR1/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 10; a concatameric fusion monomeric protein mgTNFR2-TNFR2/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 12; a concatameric fusion monomeric protein mgCD2-CD2/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 22; and a concatameric fusion monomeric protein mgCTLA4-CTLA4/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 24.

In a still further specific aspect of the present invention, there are provided a DNA construct encoding a concatameric fusion monomeric protein mgTNFR1-TNFR1/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 9; a DNA construct encoding a concatameric fusion monomeric protein mgTNFR2-TNFR2/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 11; a DNA construct encoding a concatameric fusion monomeric protein mgCD2-CD2/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 21; and a DNA construct encoding a concatameric fusion monomeric protein mgCTLA4-CTLA4/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 23. In order to produce a glycosylation motif peptide, a primer set (forward and reverse primers) is designed, which are complementary to a nucleotide sequence corresponding to the joint region between soluble extracellular domains of concatameric fusion proteins of TNFR/Fc, CD2/Fc and CTLA4/Fc, as well as containing codons encoding asparagine (N) (ATT and AAC) or codons encoding serine (S) and threonine (T) (TCC; and ACC, ACG and ACA, respectively), with which any codon in the concatameric fusion protein gene may be substituted. When designing the primer, selection of one among a plurality of amino acid sequences may be determined

depending on a condition allowing minimum substitution of the nucleotide sequence and melting temperature (T_m) of each primer.

In a still further specific aspect of the present invention, there are provided a recombinant expression plasmid pTR11Ig-MG operably linked to a DNA construct encoding a concatameric fusion monomeric protein mgTNFR1-TNFR1/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 9; a recombinant expression plasmid pTR22Ig-MG operably linked to a DNA construct encoding a concatameric fusion monomeric protein mgTNFR2-TNFR2/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 11; a recombinant expression plasmid pCD22Ig-MG operably linked to a DNA construct encoding a concatameric fusion monomeric protein mgCD2-CD2/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 21,; and a recombinant expression plasmid Pct44Ig-MG operably linked to a DNA construct encoding a concatameric fusion monomeric protein mgCTLA4-CTLA4/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 23. The recombination expression plasmids are deposited in Korean Culture Center of Microorganisms (KCCM) and are assigned accession Nos. KCCM-10404, KCCM-10407, KCCM-10401 and KCCM-The KCCM deposit will be maintained under the terms of the 10399, respectively. Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

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In a still further specific aspect of the present invention, there are provided a mammalian host cell transformed or transfected with a recombinant expression plasmid pTR11Ig-MG operably linked to a DNA construct encoding a concatameric fusion monomeric protein mgTNFR1-TNFR1/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 9; a mammalian host cell transformed or transfected with a recombinant expression plasmid pTR22Ig-MG operably linked to a DNA construct encoding a concatameric fusion monomeric protein mgTNFR2-TNFR2/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 11; a mammalian host cell transformed or transfected with a recombinant expression plasmid pCD22Ig-MG operably linked to a DNA construct encoding a concatameric fusion monomeric protein mgCD2-CD2/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 21; and a mammalian host cell transformed or transfected with a recombinant expression plasmid

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Pct44Ig-MG operably linked to a DNA construct encoding a concatameric fusion monomeric protein mgCTLA4-CTLA4/Fc containing glycosylation motif peptides, designated by SEQ ID NO: 23.

The concatameric fusion dimeric proteins of the present invention may be isolated from culture medium after culturing the transformants or transfectants according The concatameric fusion dimeric proteins may participate in to the present invention. immune response, as described in Table 1, above, and are thus useful as therapeutic agents, diagnostic agents and laboratory tools according to the kinds of the protein, and their use is well known to those of ordinary skill in the art. In particular, when being used as therapeutic agents, the concatameric fusion dimeric proteins may be applied at an therapeutically effective amount common in the art, and it will be understood that such an amount may vary depending on diverse factors including activity of the used compound, patient's age, body weight, health state, sex and diet, administration time, administration route, combination of drugs, and pathogenic state of a specific disease to be prevented or treated. In addition, when being used as therapeutic agents, it will be understood that the concatameric fusion dimeric proteins according to the present invention may be applied by the typical methods and routes for administration of proteins involving immune response, which are known to those of ordinary skill in the art.

The present invention will be explained in more detail with reference to the following examples in conjunction with the accompanying drawings. However, the following examples are provided only to illustrate the present invention, and the present invention is not limited to them. For convenience in describing the present invention, information on DNA constructs, recombinant expression plasmids and transformed cell lines, which are prepared according to the Examples, below, and the used primers and accession numbers is summarized in Tables 8 and 9, below.

TABLE 8
Information on DNA constructs and accession Nos.

DNA construct name	SEO ID No.		Deposition of genes		Deposition of cell lines	
DNA construct name	DNA	Protein	Designation	Accession No.	Designation	Accession No.
TNFR1-IgG	1	2				
INFR2-IgG	3	4		71.001.1.10000	TR11Ig-	KCLRF-BP-
TNFR1-TNFR1-IgG	5	6	pTR111g-Top10'	K.CCM 10288	CHO	00046
TNFR2-TNFR2-IgG	7	8	pTR221g-Top10'	KCCM 10291	TR22Ig- CHO	KCLRF-BP- 00049
mgTNFR1-TNFR1-IgG	9	10	pTR11Ig-MG	KCCM 10404		
mgTNFR2-TNFR2-IgG	11	12	PTR22Ig-MG	KCCM 10407		
CD2-IgG	13	14				
CTLA4-IgG	15	16				
CD2-CD2-IgG	17	18	pCD221g	KCCM 10402		
CTLA4-CTLA4-IgG	19	20	pCT44Ig	KCCM 10400		
mgCD2-CD2-IgG	21	22	pCD22Ig-MG	KCCM 10401		
mgCTLA4-CTLA4-IgG	23	24	pCT44Ig-MG	KCCM 10399		

TABLE 9
Information for primers

	Information for primers				
Primer name	SEQ	Description			
	ID No.				
Oligo TNFR-EDF- EcoRI	25	Containing 5' end of the extracellular domain of TNFR1 and an EcoRI site			
Oligo TNFR-EDR- IgGh	26	Reverse primer containing 3' end of the extracellular domain of TNFR1 and the hinge region of IgG			
Oligo IgG1-T1F	27	Containing 5' end of the hinge region of IgG and 3' end of TNFR1			
Oligo IgG1-R-XbaI	28	Reverse primer containing 3' end of the hinge region of IgG and a Xbal site			
Oligo TNFR2-EDF- EcoRI	29	Containing 5' end of the extracellular domain of TNFR2 and an EcoRI site			
Oligo TNFR2-EDR- IgGh	30	Reverse primer containing 3' end of the extracellular domain of TNFR2 and the hinge region of IgG			
Oligo IgG1-T2F	31	Containing 5' end of the hinge region of IgG and 3' end of TNFR2			
Oligo TNFR1-CF- BamHI	32	Containing 5' end of the extracellular domain of TNFR1 and a BamHI site; and used for preparation of a concatamer			
Oligo TNFR1-NR- BamHI	33	Reverse primer containing 3' end of the extracellular domain of TNFR1 and a BamHI site; and used for preparation of a concatamer			
Oligo TNFR2-CF- BamHI	34	Containing 5' end of the extracellular domain of TNFR2 and a BamHI site; and used for preparation of a concatamer			
Oligo TNFR2-NR- BamHI	35	Reverse primer containing 3' end of the extracellular domain of TNFR2 and a BamHI site; and used for preparation of a concatamer			
Oligo mgTNFR1- TNFR1-IgG-F	36	Primer for mutagenesis, containing a sequence capable of inserting glycosylation sites into the joint region of TNFR1-TNFR1, and sequences corresponding to 3' end and 5' end of TNFR1; and used for preparation of a MG (multiglycosylation) form			
Oligo mgTNFR1- TNFR1-IgG-R	37	Reverse primer for mutagenesis, containing a sequence capable of inserting glycosylation sites into the joint region of TNFR1-TNFR1, and sequences corresponding to 3' end and 5' end of TNFR1; and used for preparation of a MG form			
Oligo mgTNFR2- TNFR2-IgG-F	38	Primer for mutagenesis, containing a sequence capable of inserting glycosylation sites into the joint region of TNFR2-TNFR2, and sequences corresponding to 3' end and 5' end of TNFR2; and used for preparation of a MG form			
Oligo mgTNFR2- TNFR2-IgG-R	39	Reverse primer for mutation, containing a sequence capable of inserting glycosylation sites into the joint region of TNFR2-TNFR2, and sequences corresponding to 3' end and 5' end of TNFR2; and used for preparation of a MG form			
Oligo CD2F-EcoRI	40	Containing 5' end of the extracellular domain of CD2 and a EcoRI site			
Oligo CD2R-RstI	41	Containing 3' end of the extracellular domain of CD2 and a PstI site			
Oligo IgG-F-PstI	42	Containing 5' end of the hinge region of IgG and a PstI site			
Oligo CTLA4F-EcoRI	43	Containing 5' end of the extracellular domain of CTLA-4 and a EcoRI site			
Oligo CTLA4R-PstI	44	Containing 3' end of the extracellular domain of CTLA-4 and a PstI site			
Oligo CD2-NT-F	45	Containing 5' end of the extracellular domain of CD2; and used for preparation of a concatamer			
Oligo CD2-CT-R	46	Reverse primer containing 3' end of the extracellular domain of CD2; and used for preparation of a concatamer			
Oligo CTLA4-NT-F	47	Containing 5' end of the extracellular domain of CTLA-4; and used for preparation of a concatamer			
Oligo CTLA4-CT-R	48	Reverse primer containing 3' end of the extracellular domain of CTLA-4; and used for preparation of a concatamer			
Oligo mgCD2-CD2- IgG-F	49	Used for preparation of a MG (multiglycosylation) form of CD2-CD2-IgG			
Oligo mgCD2-CD2- IgG-R	50	Reverse primer used for preparation of a MG (multiglycosylation) form of CD2-CD2-IgG			
Oligo mgCTLA4- CTLA4-IgG-F	51	Used for preparation of a MG (multiglycosylation) form of CTLA4-CTLA4-IgG			
Oligo mgCTLA4- CTLA4-IgG-R	52	Reverse primer used for preparation of a MG (multiglycosylation) form of CTLA4-CTLA4-IgG			

EXAMPLE 1

Human TNFR

A. Manufacture of a DNA construct encoding simple fusion monomeric protein of TNFR1/Fc (Fig. 1 and Fig. 5)

a. DNA fragment encoding soluble extracellular domain of TNFR1

A fusion gene encoding soluble extracellular domain of type I human TNF receptor (TNFR1, p55) and Fc fragment of human immunoglobulin G1 was constructed by the Polymerase Chain Reaction (PCR) method described in the prior art (Holten et al., Biotechniques 8:528, 1990).

A DNA fragment encoding soluble extracellular domain of TNFR1 was constructed by PCR using a primer (the sequence of nucleotide of SEQ ID NO: 25) with EcoRI restriction site and the sequence encoding leader sequence (the sequence of amino acids 1-20 of SEQ ID NO: 2), and an antisense primer (the sequence of nucleotide of SEQ ID NO: 26) with the sequence encoding a part of 3' ends of the said soluble extracellular domain of TNFR1 (TNFR1-ED) and 5' ends of the hinge region of immunoglobulin G1 (IgG1). The template cDNA for this reaction was constructed by reverse transcription PCR (RT-PCR) of mRNA extracted from monocyte (T lymphocyte) of healthy adults.

After blood of healthy adults was extracted and diluted to 1:1 with RPMI-1640 (Gibco BRL, USA), the layer of T lymphocyte which formed at upper part was obtained by density gradient centrifugation using Ficoll-hypaque (Amersham, USA). In order to make the concentration of the cell to 5X10⁵ cells/ml, the cell was washed with RPMI-1640 for 3 times, and RPMI-1640 culture media containing 10% Fetal Bovine Serum (FBS, Gibco BRL, USA) was added, then cultured at 37 °C for two days in the 5% CO₂ incubator after adding leukoagglutinin to 3.5ug/ml (Pharmacia, USA).

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The mRNAs were purified using Tri-Reagent (MRC, USA) mRNA purification kit. First, 2X10⁷ of human T lymphocyte was washed with Phosphate Buffered Saline (PBS, pH7.2) for 3 times, and then 1ml of Tri-Reagent was mixed for several times to dissolve RNA. After adding 0.2ml of chloroform to this tube and mixing thoroughly, this tube was incubated at room temperature (RT) for 15 min, then centrifuged at 15,000 rpm, 4°C for 15 min. The upper part of the solution was transferred to a 1.5ml tube, and 0.5ml of isopropanol was added, and then centrifuged at 15,000 rpm, 4°C for 15 min. After the supernatant was discarded, the pellet was resuspended with 1ml of 3° distilled water treated with 75% ethanol-25% DEPC (Sigma, USA), and then centrifuged at 15,000 rpm, 4°C for 15 min. After the supernatant was removed completely and dried in the air to remove ethanol residue, RNA was resuspended with 50µl of 3° distilled water treated with DEPC.

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The primary cDNA was synthesized by mixing 2µg of purified mRNA and 1µl of oligo dT (dT30, Promega, USA) primer to 10µM in 1.5ml tube, heating at 70°C for 2 min, and cooling in ice for 2 min. After that, this mixture was added with 200U of M-MLV reverse transcriptase (Promega, USA), 10µl of 5 x reaction buffer (250mM Tris-HCl, pH 8.3, 375mM KCl, 15mM MgCl₂, and 50mM DTT), 1µl of dNTP (10mM each, Takara, Japan), and DEPC-treated 3° distilled water to 50µl, then reacted at 42°C for 1 hour.

b. DNA fragment encoding Fc fragment of immunoglobulin

A DNA fragment encoding Fc fragment of immunoglobulin G1 was constructed by PCR using a primer (the sequence of nucleotide of SEQ ID NO: 27) with the sequence encoding a part of 3' ends of the said soluble extracellular domain of TNFR and 5' end of the hinge region of immunoglobulin G1 (IgG1), and an antisense primer (the sequence of nucleotide of SEQ ID NO: 28) with XbaI restriction site and the sequence encoding 3' ends of IgG1 Fc. The template cDNA for this reaction was constructed by RT-PCR of mRNA extracted from peripheral blood cell (B lymphocyte) of convalescent patients with pyrexia of unknown origin.

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c. DNA construct encoding simple fusion monomeric protein of TNFR1/Fc

After DNA fragment encoding soluble extracellular domain of TNFR1 and DNA fragment encoding Fc fragment of immunoglobulin produced as described above were mixed in the same tube, complementary binding between the common sequence (the sequence including 3' end of soluble extracellular domain of TNFR1 and 5' end of IgG1 hinge region) was induced. Using this mixture as a template, DNA construct including DNA fragment encoding soluble extracellular domain of TNFR1 and DNA fragment encoding IgG1 Fc fragment was amplified by PCR using a primer (the sequence of nucleotide of SEQ ID NO: 25) with the sequence encoding 5' end of TNFR1 and another primer (the sequence of nucleotide of SEQ ID NO: 28) with the sequence encoding 3' end of IgG1 Fc. The constructed gene included a leader sequence to faciliate secretion of protein after expression.

d. Cloning of the DNA construct encoding simple fusion monomeric protein of TNFR1/Fc

DNA construct encoding simple fusion monomeric protein of TNFR1/Fc as described above was restricted with EcoRI and XbaI, and cloned by inserting into a commercially available cloning vector, pBluescript KS II (+) (Stratagene, USA), at EcoRI/XbaI site. The sequence of a total coding region was identified by DNA sequencing (SEQ ID NO: 1). This produced fusion protein was designated TNFR1/Fc as simple fusion monomeric protein, and the elliptical shape shown in Figure 1 represents the structure of a primary expression product of the fusion gene. The deduced amino acid sequence of simple fusion monomeric of TNFR1/Fc corresponded to SEQ ID NO: 2.

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B. Manufacture of a DNA construct encoding simple fusion monomeric protein of TNFR2/Fc (Fig. 1 and Fig. 5)

a. DNA fragment encoding soluble extracellular domain of TNFR2

A fusion gene encoding soluble extracellular domain of type II human TNF receptor (TNFR2, p75) and Fc fragment of human immunoglobulin G1 was constructed by the same method as that of TNFR1/Fc.

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A DNA fragment encoding soluble extracellular domain of TNFR2 was constructed by PCR using a primer (the sequence of nucleotide of SEQ ID NO: 29) with EcoRI restriction site and the sequence encoding leader sequence (the sequence of amino acids 1-22 of SEQ ID NO: 4), and an antisense primer (the sequence of nucleotide of SEQ ID NO: 30) with the sequence encoding a part of 3' ends of said soluble extracellular domain of TNFR2 (TNFR2-ED) and 5' ends of the hinge region of immunoglobulin G1 (IgG1). The template cDNA for this reaction was constructed by RT-PCR of mRNA extracted from monocyte (T lymphocyte) of healthy adults.

b. DNA construct encoding simple fusion monomeric protein of TNFR2/Fc

After DNA fragment encoding soluble extracellular domain of TNFR2 and DNA fragment encoding Fc fragment of immunoglobulin G1 produced as described above were mixed in the same tube, complementary binding between the common sequence (the sequence including 3' end of soluble extracellular domain of TNFR2 and 5' end of IgG1 hinge region) was induced. Using this mixture as a template, DNA construct including DNA fragment encoding soluble extracellular domain of TNFR2 and encoding and DNA fragment encoding IgG1 Fc fragment was amplified by PCR using a primer (the sequence of nucleotide of SEQ ID NO: 29) with the sequence encoding 5' end of TNFR2 and another primer (the sequence of nucleotide of SEQ ID NO: 28) with the sequence encoding 3' end of IgG1 Fc. The constructed gene includes a sequence to faciliate secretion of protein after expression.

c. Cloning of the DNA construct encoding simple fusion monomeric protein of TNFR2/Fc

DNA construct encoding simple fusion monomeric protein of TNFR2/Fc as described above was restricted with EcoRI and XbaI, and cloned by inserting into a commercially available cloning vector, pBluescript KS II (+) (Stratagene, USA), at EcoRI/XbaI site. The sequence of a total coding region was identified by DNA sequencing (SEQ ID NO: 3). This produced fusion protein was designated TNFR2/Fc as simple fusion monomeric protein, and the elliptical shape shown in Figure 1 represents the structure of a primary expression product of the fusion gene. The deduced amino acid sequence of simple fusion monomeric of TNFR2/Fc corresponded to SEQ ID NO: 4.

C. Manufacture of a DNA construct encoding concatameric fusion monomeric protein of TNFR1-TNFR1/Fc (Fig. 2 and Fig. 5)

In order to manufacture a fusion gene comprising the concatameric shape in soluble extracellular domain of TNFR1, i.e. the DNA construct encoding concatameric fusion monomeric protein of TNFR1-TNFR1/Fc, BamHI restriction site was inserted respectively into the sequence of soluble extracellular domain of TNFR1 and DNA construct as produced as above encoding simple fusion monomeric protein of TNFR1/Fc by PCR, and then regions of each fragments restricted by BamHI were linked by ligase. The DNA construct, encoding simple fusion monomeric protein of TNFR1/Fc produced as above, was used as the template of this reaction.

The fragment of the soluble extracellular domain of TNFR1 with BamHI restriction site at 3' end was amplified by PCR using a primer corresponding to the nucleotide of SEQ ID NO: 25 and another primer corresponding to the nucleotide sequence of SEQ ID NO: 33, and the other fragment of simple fusion monomeric protein of TNFR1/Fc with BamHI restriction site at 5' end was amplified by PCR using a primer

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corresponding to the nucleotide of SEQ ID NO: 28 and another primer corresponding to the nucleotide sequence of SEQ ID NO: 32, respectively. PCR was performed by adding 1µl of primary cDNA, 2U of Pfu DNA polymerase (Stratagene, USA), 10µl of 10X reaction buffer [200mM Tris-HCl, pH 8.75, 100mM (NH₄)₂SO₄, 100mM KCl, 20mM MgCl₂], 1% TritonTM X-100, 1mg/ml BSA, 3µl primer 1 (10µM), 3µl primer 2 (10µM), 2µl dNTP (10mM each), and 3° distilled water to 100µl. The reaction condition was as follows; 94°C, 5 min; 95°C, 1 min; 58°C, 1 min 30 sec; 72°C, 1 min for 31 cycles; and 72°C, 15 min to make PCR product with complete blunt end.

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After electrophorized on 0.8% agarose gel, the PCR product was purified by Qiaex II gel extraction kit (Qiagen, USA). The purified PCR product was restricted by BamHI and extracted by phenol-chloroform extraction methods. Subsequently, two kinds of DNA fragments restricted by BamHI were linked by ligase.

D. Manufacture of a DNA construct encoding concatameric fusion monomeric protein of TNFR2-TNFR2/Fc (Fig. 2 and Fig. 5)

After a BamHI restriction site was inserted respectively into the sequence of the soluble extracellular domain of TNFR21 and the DNA construct produced as described above encoding simple fusion monomeric protein of TNFR2/Fc by PCR, a DNA construct encoding concatameric fusion monomeric protein of TNFR2-TNFR2/Fc was manufactured by linking the regions of each fragments restricted by BamHI by ligase.

A fragment of soluble extracellular domain of TNFR2 with BamHI restriction site at 3' end was amplified using a primer corresponding the sequence of SEQ ID NO: 34 and SEQ ID NO: 35. PCR was performed as that of TNFR1, except that a DNA construct encoding simple fusion monomeric protein of SEQ ID NO: 3 produced as above was used as a template. The PCR product was purified by the method as that of TNFR1.

E. DNA construct encoding concatameric fusion monomeric protein of TNFR1-TNFR1/Fc with glycosylation motif.

A DNA fragment was manufactured by PCR using an antisense primer (the sequence of nucleotide of SEQ ID NO: 37) with the sequence encoding the part (the sequence of nucleotide 565-591 of SEQ ID NO: 5) of 3' end of the first soluble extracellular domain of TNFR1, except the sequence of hydrophobic peptide region (the sequence of amino acid 197-216 of SEQ ID NO: 6) at the junction of soluble extracellular domain of TNFR1 and the part (the sequence of nucleotide 649-681 of SEQ ID NO: 5) of 5' end of the second soluble extracellular domain of TNFR1, and another primer (the sequence of nucleotide of SEQ ID NO: 25) with the sequence encoding EcoRI restriction site and leader sequence.

In addition, the total four amino acid sequences encoding glycosylation site (the sequence of amino acids 189-191, 192-194, 198-200, and 204-206 of SEQ ID NO: 10) were inserted by manufacturing the primer as above (the sequence of nucleotide of SEQ ID NO: 36 and 37) corresponding the substitution of the nucleotide 565-567 (CTG, Leu), 574-576 (ACG, Thr), 652-654 (CTA, Leu), and 670-672 (AGA, Arg) of SEQ ID NO: 5 with the nucleotide of AAC (Asn, N); the nucleotide of 571-573 (TGC, Cys) and 580-582 (TTG, Leu) of SEQ ID NO: 5 with the nucleotide of ACC (Thr, T); the nucleotide of 658-660 (GAC, Asp) with the nucleotide of TCC (Ser, S).

In this reaction, the gene (the nucleotide of SEQ ID NO: 5) encoding concatameric shape of TNFR1-TNFR1/Fc was used as a template. During the primary PCR, only the half of the antisense primer was induced to bind the gene encoding concatameric shape of TNFR1-TNFR1/Fc used as a template, and, as chain reaction was proceeding, the unbound part to the template was induced to form a complete double-stranded DNA by polymerase, and then this was capable of producing the DNA fragment with state of linkage of the sequence of 5' end encoding the part of the second soluble

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extracellular domain and the sequence of 3' end encoding TNFR1 extracellular domain including leader sequence. Therefore, a part of the sequence of 5' end encoding the second soluble extracellular domain has the function that was capable of binding to the second DNA fragment as follows.

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The second DNA fragment was manufactured by PCR using a primer (the sequence of nucleotide of SEQ ID NO: 36) with the sequence encoding the part (the sequence of nucleotide 565-591 of SEQ ID NO: 5) of 3' end of the first soluble extracellular domain of TNFR1 and the part (the sequence of nucleotide 649-681 of SEQ ID NO: 5) of 5' end of the second soluble extracellular domain of TNFR1, and an antisense primer (the sequence of nucleotide of SEQ ID NO: 28) with the sequence encoding a XbaI restriction site and 3' end of IgG1 Fc. This reaction was also performed as described above, that is, only the half of antisense primer was induced to bind the template, and consequently, DNA fragment like that described above had the sequence encoding 5' end of TNFR1 extracellular including the part of 3' end of the first soluble extracellular domain.

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Subsequently, resulting from two kinds of DNA fragments as PCR described as above were mixed in the same tube, induced to bind between common sequences, and fused by PCR using primers (the sequence of nucleotide of SEQ ID NO: 25 and 28) encoding 5' and 3' end of each concatameric genes, and the product was designated mgTNFR1-TNFR1-IgG.

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F. DNA construct encoding concatameric fusion monomeric protein of TNFR2-TNFR2/Fc with glycosylation motif.

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A DNA fragment was manufactured by PCR using an antisense primer (the sequence of nucleotide of SEQ ID NO: 39) with the sequence encoding the part (the sequence of nucleotide 586-606 of SEQ ID NO: 7) of 3' end of first soluble extracellular domain of TNFR2, except the sequence of hydrophobic peptide region (the sequence of

amino acid 203-263 of SEQ ID NO: 8) at the junction of soluble extracellular domain of TNFR2 and the part (the sequence of nucleotide 790-807 of SEQ ID NO: 7) of 5' end of second soluble extracellular domain of TNFR2, and another primer (the sequence of nucleotide of SEQ ID NO: 29) with the sequence encoding EcoRI restriction site and leader sequence.

In addition, the total two amino acid sequences encoding glycosylation site (the sequence of amino acids 199-201 and 206-208 of SEQ ID NO: 12) were inserted by manufacturing the primer as described above (the sequence of nucleotide of SEQ ID NO: 38 and 39) corresponding to the substitution of the nucleotide 595-597 (GTC, Val) and 799-801 (GGG, Gly) SEQ ID NO: 7 with the nucleotide of AAC (Asn, N).

In this reaction, the gene (the nucleotide of SEQ ID NO: 7) encoding concatameric shape of TNFR2-TNFR2/Fc was used as a template. During the primary PCR, only the half of antisense primer was induced to bind the gene encoding concatameric shape of TNFR2-TNFR2/Fc used as a template, and, as the chain reaction was proceeding, the unbound part to the template was induced to form a complete double-stranded DNA by polymerase, and thus this was capable of producing the DNA fragment with a state of linkage of the sequence of 5' end encoding the part of the second soluble extracellular domain and the sequence of 3' end encoding TNFR2 extracellular domain including the leader sequence. Therefore, a part of the sequence of 5' end encoding the second soluble extracellular domain has the function that was capable of binding to the second DNA fragment as follows.

The second DNA fragment was manufactured by PCR using a primer (the sequence of nucleotide of SEQ ID NO: 38) with the sequence encoding the part (the sequence of nucleotide 586-606 of SEQ ID NO: 7) of 3' end of the first soluble extracellular domain of TNFR2 and the part (the sequence of nucleotide 790-807 of SEQ ID NO: 7) of 5' end of the second soluble extracellular domain of TNFR2, and an antisense primer (the sequence of nucleotide of SEQ ID NO: 28) with the sequence encoding a XbaI restriction

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site and 3' end of IgG1 Fc. This reaction was also performed, that is, only the half of antisense primer was induced to bind the template, and consequently, DNA fragment like that described above had the sequence encoding 5' end of TNFR2 extracellular including the part of 3' end of first soluble extracellular domain.

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Subsequently, resulting from two kinds of DNA fragments as PCR produced as above were mixed in the same tube, induced to bind between common sequences, and fused by PCR using primers (the sequence of nucleotide of SEQ ID NO: 29 and 28) encoding 5' and 3' end of each concatameric genes, and the product was designated mgTNFR2-TNFR2-IgG.

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G. Cloning of DNA constructs encoding concatameric fusion monomeric protein of TNFR-TNFR/Fc and their glycosylated forms

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DNA constructs encoding concatameric fusion monomeric protein of TNFR-TNFR/Fc and their glycosylated forms as above were cloned by inserting into pBluescript KS II (+) (Stratagene, USA) at EcoRI/XbaI site. These produced fusion proteins were designated TNFR1-TNFR1/Fc and TNFR2-TNFR2/Fc as concatameric fusion monomeric protein, and designated mgTNFR1-TNFR1/Fc and mgTNFR2-TNFR2/Fc as their glycosylated forms. The deduced amino acid sequences corresponded to SEQ ID NO: 6, 8, 10, and 12.

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After 10µg of pBluescript KS II (+) (Stratagene, USA) used as a vector was mixed with 15U of EcoRI, 15U of XbaI, 5µl of 10X reaction buffer (100mM Tris-HCl, pH 7.5, 100mM MgCl₂, 10mM DTT, 500nM NaCl), 5µl of 0.1% BSA (Takara, Japan), and 3° distilled water to 50µl, DNA was restricted by incubation at 37°C for 2 hrs. After electrophorized on 0.8% agarose gel, the PCR product was purified by Qiaex II gel extraction kit (Qiagen, USA).

After 100ng of pBluescript KS II (+) (Stratagene, USA) restricted by EcoRI and XbaI was mixed with 20ng of PCR product restricted by the restriction enzyme, 0.5U of T4 DNA ligase (Amersham, USA), 1µI of 10X reaction buffer (300mM Tris-HCl, pH 7.8, 100mM MgCl₂, 100mM DTT, 10mM ATP) and 3° distilled water were added to 10µI, and the mixture was incubated in the water bath at 16°C for 16 hrs. E. coli Top10 (Novex, USA) was made to competent cell by the method of rubidium chloride (RbCl, Sigma, USA) and transformed, then spread on the solid LB media including 50µg/ml of ampicillin (Sigma, USA) and incubated at 37°C for 16 hrs. Formed colonies were inoculated in 4ml of liquid LB media including 50µg/ml of ampicillin and incubated at 37°C for 16 hrs. Plasmid was purified by the method of alkaline lysis according to Sambrook et al. (Molecular cloning, Cold Spring Harbor Laboratory press, p1.25-1.31, p1.63-1.69, p7.26-7.29, 1989) from 1.5ml of that, and the existence of cloning was confirmed by the restriction of EcoRI and XbaI.

The sequence of a total coding region was identified by the DNA sequencing method of dideoxy chain termination method (Sanger et al., Proc. Natl. Acad. Sci., 74:5483, 1977) as follows. The DNA sequencing reaction was performed according to the manual using a plasmid purified by alkaline lysis method as described above and SequenaseTM ver 2.0 (Amersham, USA). After the reaction mixture as above was loaded on 6% polyacrylamide gel and electrophorized for 2 hrs at constant voltage of 1,800~2,000 V and 50 °C, DNA sequence was identified by exposing to X-ray film (Kodak, USA) after the gel was dried out.

EXAMPLE 2 AND 3

CD2 and CTLA4

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DNA fragments encoding soluble extracellular domain of CD2 and CTLA4 were constructed by PCR using a primer [CD2(the sequence of nucleotide of SEQ ID NO:

40), and CTLA4(the sequence of nucleotide of SEQ ID NO: 43)] with EcoRI restriction site and the coding sequence [CD2 (the sequence of nucleotide of SEQ ID NO: 13), and CTLA4 (the sequence of nucleotide of SEQ ID NO: 15)] encoding the leader sequence [CD2(the sequence of amino acid 1-24 of SEQ ID NO: 14), and CTLA4(the sequence of amino acid 1-21 of SEQ ID NO: 16)], and an antisense primer [CD2(the sequence of nucleotide of SEQ ID NO: 41), and CTLA4(the sequence of nucleotide of SEQ ID NO: 44)] with PstI restriction site and the sequence [CD2(the sequence of nucleotide of SEQ ID NO: 13), and CTLA4(the sequence of nucleotide of SEQ ID NO: 15)] encoding 3' end of the soluble extracellular domain of the proteins as described above. The template cDNA for this reaction was constructed by reverse transcription PCR (RT-PCR) of mRNA extracted from the monocyte (T lymphocyte) of healthy adults.

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Also, a DNA fragment encoding Fc fragment of immunoglobulin G1 was constructed by PCR using a primer (the sequence of nucleotide of SEQ ID NO: 42) with PstI restriction site and the sequence encoding 5' ends of constant region of IgG1, and an antisense primer (the sequence of nucleotide of SEQ ID NO: 28) with XbaI restriction site 'and the sequence encoding 3' ends of IgG1 Fc. The template cDNA for this reaction was constructed by RT-PCR of mRNA extracted from peripheral blood cell (B lymphocyte) of convalescent patients with unknown fever.

Subsequently, both DNA fragment encoding soluble extracellular domain of CD2 and CTLA4 and DNA fragment encoding Fc fragment of immunoglobulin G1 produced as described above were restricted by PstI, and then the simple dimeric shape of CD2/Fc and CTLA4/Fc genes were constructed by linkages using T4 DNA ligase. The constructed genes included a leader sequence to faciliate secretion of protein after expression.

DNA constructs as described above were restricted by restriction enzyme of EcoRI and XbaI, and cloned by inserting into a commercially available cloning vector, pBluescript KS II (+) (Stratagene, USA) at EcoRI/XbaI site. The sequence of a total coding

region was identified by DNA sequencing (SEQ ID NO: 13 and 15). These produced fusion proteins were designated CD2/Fc and CTLA4/Fc, and the deduced amino acid sequences of these corresponded to SEQ ID NO: 14 and 16.

PCR was performed by adding 1μl of primary cDNA, 2U of Pfu DNA polymerase (Stratagene, USA), 10μl of 10X reaction buffer [200mM Tris-HCl, pH 8.75, 100mM (NH₄)₂SO₄, 100mM KCl, 20mM MgCl₂], 1% TritonTM X-100, 1mg/ml BSA, 3μl primer 1 (10μM), 3μl primer 2 (10μM), 2μl dNTP (10mM each), and 3° distilled water to 100μl. The reaction condition was as follows; 94°C, 5 min; 95°C, 1 min; 58°C, 1 min 30 sec; 72°C, 1 min for 31 cycles; and 72°C, 15 min to make PCR product with complete blunt end.

The fusion genes with concatameric shape of CD2-CD2/Fc and CTLA4-CTLA4/Fc were constructed as follows.

In order to manufacture fusion gene comprising the concatameric shape in soluble extracellular domain of CD2 and CTLA4, the sequences of soluble extracellular domain of CD2 and CTLA4 were inserted by blunt-end ligation using ligase at the junction between extracellular domain and immunoglobulin of fusion genes in the shape of simple dimer with blunt end, using PstI restriction enzyme and T4 DNA polymerase. Specifically, DNA constructs were constructed by PCR using a primer [CD2(the sequence of nucleotide of SEQ ID NO: 13) and CTLA4(the sequence of nucleotide of SEQ ID NO: 48)] with the coding sequence [CD2(the sequence of nucleotide of SEQ ID NO: 13) and CTLA4(the sequence of micleotide of SEQ ID NO: 15)] encoding the end of leader sequence [CD2(the sequence of amino acid 25 of SEQ ID NO: 14) and CTLA4(the sequence of amino acid 22 of SEQ ID NO: 16)] of soluble extracellular domain, and an antisense primer [CD2(SEQ ID NO: 46) and CTLA4(SEQ ID NO: 48)] with the sequence [CD2(the sequence of nucleotide of SEQ ID NO: 13) and CTLA4(the sequence of nucleotide of SEQ ID NO: 15)] encoding 3' end of soluble extracellular domain as above. The simple fusion monomeric genes [CD2/Fc (the sequence of nucleotide of SEQ ID NO: 13) and CTLA4/Fc (the sequence of

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nucleotide of SEQ ID NO: 15)] described as above were used as the template of this reaction.

Also, CD2/Fc and CTLA4/Fc, which were inserted in pBluescript KS II (+) in the shape of simple monomeric form, were made to have 3' overhang end using the restriction enzyme of PstI. The cut end of 3' overhang was partially deleted to form a blunt end by treating T4 DNA polymerase. In order to manufacture fusion genes in the shape of concatamer in soluble extracellular domain, the soluble extracellular domains of CD2 and CTLA4 produced by PCR as described above were cloned by inserting into cut ends of simple monomeric gene made as blunt end. These produced fusion proteins were designated CD2-CD2/Fc and CTLA4-CTLA4/Fc as concatameric fusion monomeric protein, and their deduced amino acid sequences corresponded SEQ ID NO: 18 and 20, respectively.

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The concatameric fusion genes in the shape of multiglycosylated form were constructed as follows.

The glycosylation mofit was inserted by secondary PCR with mixing in the same tube of a DNA fragment produced by PCR using a primer including EcoRI restriction site and the soluble extracellular domain with leader sequence, and an antisense primer with the sequence encoding the part of 3' end of the first soluble extracellular domain of concatameric shape of fusion gene and the part of 5' end of the second soluble extracellular domain with the nucleotide of substituted glycosylation motif; and other DNA fragment produced by PCR using a primer with the sequence encoding the part of 3' end of the first soluble extracellular domain of concatameric shape of fusion gene and the part of 5' end of the second soluble extracellular domain with the nucleotide of substituted glycosylation motif, and an antisense primer with the sequence encoding 3' end of Fc fragment of immunoglobulin G1 and XbaI restriction site.

In the case of concatameric fusion gene of CD2/Fc and CTLA4/Fc, the glycosylation motif was inserted by PCR using modified primers as the same methods as

that of TNFR/Fc described as above, but it was different from the case of TNFR/Fc that the amino acid sequence of binding to soluble extracellular domain of CD2 and CTLA4 was retained as the same.

In the process of multiglycosylatin of the concatameric fusion protein of CD2/Fc and CTLA4/Fc, the case of CD2/Fc was completed by inserting the total two glycosylation motif peptide region (the sequence of amino acid of 200-202 and 206-208 of SEQ ID NO: 22) using a manufactured primer including the substitution of the nucleotide of 598-600 (CCT, Pro) and 616-618 (GAG, Glu) of SEQ ID NO: 17 with AAT (Asn, N), and the case of CTLA4/Fc was completed by inserting the total three glycosylation motif peptide region (the sequence of amino acid of 136-138, 142-144, and 147-149 of SEQ ID NO: 24) using a manufactured primer(SEQ ID NO: 51 and 52) including the substitution of the nucleotide of 403-405 (GTA, Val) and 424-426 (CCA, Pro) of SEQ ID NO: 19 with AAT (Asn, N); the nucleotide of 409-411 (GAT, Asp) and 445-447 (GTG, Val) with ACA (Thr, T) and ACG (Thr, T), respectively. These produced fusion proteins were designated mgCD2-CD2/Fc and mgCTLA4-CTLA4/Fc as concatameric fusion monomeric protein, and their deduced amino acid sequences corresponded to SEQ ID NO: 22 and 24, respectively.

EXAMPLE 4

Expression and purification of simple/concatameric fusion dimeric protein of <u>TNFR/Fc</u>

In order to express the fusion proteins in CHO-K1 cell (ATCC CCL-61, Ovary, Chinese hamster, Cricetulus griseus), after pBluescript KS II (+) plasmid DNA including TNFR/Fc fusion gene was purified from transformed E. coli, an animal cell expression vectors were constructed as TNFR/Fc fragment produced by restriction using EcoRI and XbaI was inserted at EcoRI/XbaI site of an animal cell expression vector, pCRTM3

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(Invitrogen, USA) plasmid. And these were designated plasmid pTR11-Top10' and plasmid pTR22-Top10', and deposited as accession numbers of KCCM 10288 and KCCM 10291, respectively, at Korean Culture Center of Microorganisms (KCCM) on Jul. 10. 2001.

Transfection was performed by mixing either the plasmid pTR11-Top10' or plasmid pTR22-Top10' DNA including TNFR/Fc fusion genes as described above with the reagent of LipofectaminTM (Gibco BRL, USA). CHO-K1 cells with the concentration of 1~3 X 10⁵ cells/well were inoculated in 6-well tissue culture plate (Nunc, USA), and incubated to 50~80% in 10% FBS - DMEM media, then the DNA-liposome complex, which was reacted for 15~45 min with 1~2µg of either the plasmid pTR11-Top10' or plasmid pTR22-Top10' DNA including TNFR/Fc fusion genes as described above and 2~25µl of LipofectaminTM (Gibco BRL, USA), were added to the cell culture plate in the serum-free DMEM media. After incubation for 5 hrs, DMEM media with 20% serum was added and cells were incubated further for 18~24 hrs. After primary transfection, cells were incubated for 3 weeks in 10% FBS - DMEM media with 1.5mg/ml of Geneticin (G418, Gibco BRL, USA), and formed colonies was selected for amplified incubation. The expression of fusion proteins was analyzed by ELISA using a peroxidase labeled goat anti-human IgG (KPL, USA).

ELISA was performed as follows. First, 1mg/ml of a peroxidase labeled goat anti-human IgG (KPL, USA) was diluted to 1:2,000 with 0.1M sodium bicarbonate, 100 μ l of that was aliquoted into 96-well flexible plate (Falcon, USA) and sealed with plastic wrap, then incubated at 4°C over 16 hrs to be coated on the surface of the plate. After this, it was washed for 3 times with washing buffer (0.1% Tween-20 in 1X PBS) and dilution buffer (48.5ml 1XPBS, 1.5ml FBS, 50ul Tween-20), and then was aliquoted to 180l. After 20 μ l of culture supernatant was dropped in the first well, then serially diluted using a micropipette, and $0.01\mu g/\mu \ell$ of human immunoglobulin G (Sigma, USA) as the positive control and the culture media of untransfected CHO K-1 cell as the negative was equally diluted. After dilution, 96-well ELISA plate (Falcon, USA) was wrapped with aluminum

foil and incubated at 37°C for 1 hr 30 min, washed for 3 times with washing buffer. Peroxidase conjugated goat anti-human IgG (KPL, USA) was diluted to 1:5,000 with dilution buffer, aliquoted to 100µl, wrapped with aluminum foil, and reacted at 37°C for 1 hr. After reaction, this plate was washed for 3 times, colorized using TMB microwell peroxidase substrate system (KPL, USA) and existence of expression was confirmed by measurement of absorbance at 655nm wavelength using microplate reader (Bio-Rad, Model 550, Japan).

Transfectants manufactured as above were designated TR11Ig-CHO and TR22Ig-CHO and deposited as accession numbers of KCLRF-BP-00046 and KCLRF-BP-00049, respectively, at Korean Cell Line Research Foundation (KCLRF) on Jul. 7. 2001. And adaptation for transfectants as described above to one of the serum free media, CHO-S-SFM II (Gibco BRL, USA), was proceeded to purify the proteins produced by those transfectants as follows. After about 3X10⁵ of cells were inoculated into the 6-well plate, cells were cultured at 5% CO2, 37°C for over 16 hrs to adhere, and it was checked under a microscope that cells were adhered at about 30~50% area of the plate, then cells were cultured in a media consisting of 10% FBS DMEM and CHO-S-SFM II in the ratio of 8:2. After culturing 3 times serial passage at this ratio, it was cultured 3 times at the ratio of 6:4; 3 times at 4:6; 3 times at 3:7; 3 times at 2:8; 3 times at 1:9; and finally cultured in 100% CHO-S-SFM II media. And the level of expression was measured by ELISA.

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After these transfectant cells were cultured on a large scale in CHO-S-SFM II, the supernatants including each fusion proteins were centrifuged at 200X g for 12min to remove cell debris, and proteins were purified by the method using HiTrap protein A column (Amersham, USA) as follows. After 20mM of sodium phosphate (pH 7.0, Sigma, USA) was passed at the velocity of 1ml/min for 2 min, 10ml of supernatant was passed at the same velocity to bind fusion protein to protein A. After 20mM of sodium phosphate (pH 7.0) was passed at the same velocity for 2 min to wash, 500µl of the extracts were serially fractionated in a 1.5ml tube as 0.1M of citric acid (pH 3.0, Sigma, USA) was

passed at the the same velocity for 3 min. This was adjusted to pH 7.0 using 1M of Tris (pH 11.0, USB, USA), the existence of fusion proteins in tube was confirmed through ELISA as described above. The purified proteins were concentrated by centrifugation at 2000Xg, 4°C for 30min using Centricon 30 (Amicon, USA)

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Example 5.

SDS-PAGE of purified TNFR1-TNFR1/Fc and TNFR2-TNFR2/Fc (Fig. 15)

Proteins purified using protein A column were electrophorized by the method of SDS-PAGE in reducing condition added by DTT, reducing reagent (which destroy disulfide bond), and in a non-reducing condition excluding DTT. The result of the estimation of molecular weight on SDS-PAGE is shown in Table 10. It was possible to confirm that TNFR/Fc proteins were the shape of a dimer in the cell. The molecular weight deduced from the amino acid sequence of TNFR1-TNFR1-Ig was about 70kDa, and was estimated as about 102kDa on SDS-PAGE. As this difference could be regarded as a general phenomenon which generate on the electrophoresis of glycoproteins, this feature seemed to occurr as the result from decrease in mobility on the electrophoresis by the site of glycosylation.

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Table 10. Molecular weight of TNFR-TNFR/Fc on the SDS-PAGE.

Doctor	Molecular weight (kDa)		
Proteins	Reducing condition	Non-reducing condition	
TNFR1-TNFR1/Fc	102	200	
TNFR2-TNFR2/Fc	115	. 220	

Example 6.

Experiment of neutralization effect of simple/concatameric fusion dimeric TNFR/Fc fusion proteins on the cytotoxicity of TNFα and TNFβ

An L929 cell [ATCC, Mus musculus (mouse), NCTC clone 929 (derivative of strain L; L-929; L cell) was used for testing the effect of TNFR/Fc fusion protein on the inhibition of cytotoxicity induced by TNFα and TNFβ. This analysis was based on the TNFR activity of inhibiting cytotoxicity induced by TNF (Scallon et al., Cytokine 7:759, 1995).

L929 cells were inoculated to be 3×10^4 cells/well in 96-well plates, and incubated at 37°C for 24 hrs in a CO₂ incubator. Subsequently, actinomycin D (Sigma, USA) was added to 3µg/ml, and cells were incubated for 16~18 hrs with TNFα and TNFβ in the concentration of expressing 100% cytotoxicity (0.5~2ng/ml), and with serially 10 times diluted TNFR sample. Then, the cells in the 96-well plate were stained by the staining reagent, crystal violet (Wako Pure Chemical Industries, Japan) and the activity of the cells was estimated by the degree of absorbance at 595 nm wavelength using a spectrophotometer (Bio-Rad, Model-550, Japan).

As shown in Table 11 represented by IC₅₀ of each TNFR/Fc fusion protein, concatameric fusion proteins (TNFR1-TNFR1/Ig and TNFR2-TNFR2/Ig) have shown the higher inhibitory effect on the cytotoxicity induced by two kinds of TNF than simple dimeric fusion proteins (TNFR1/Ig and TNFR2/Ig). Also, as compared with the effects of existing simple fusion dimer and concatameric shaped TNFR/Fc fusion protein dimer of the present invention on the inhibition of cytotoxicity of TNFα (Fig. 16) and TNFβ (Fig. 17), it more clearly appeared that concatameric shaped TNFR/Fc fusion protein dimers of the present invention remarkably inhibited the TNFα and TNFβ cytotoxicity.

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Table 11. IC₅₀ of cytotoxicity inhibition

Fusion proteins	IC50 (ug/ml)	

		TNFα treated	TNFβ treated
Simple dimer	[TNFR1/Fc] ₂	63	129
	[TNFR2/Fc] ₂	189	469
Concatameric dimer	[TNFR1-TNFR1/Fc] ₂	9	20
	[TNFR2-TNFR2/Fc] ₂	15	15

Example 7

Experiment of suppressive effect of simple/concatameric fusion dimeric CD2/Fc fusion

protein and CTLA4/Fc fusion protein on the proliferation of active immune cell

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WT100B1S, a cell line of B lymphocyte which was made by transfection of pyrexia patient's B lymphocyte with Ebstein-Barr virus was incubated in RPMI 1640 supplemented with 10% FBS to use as antigen presenting cell of T lymphocyte. After centrifuged at 2,000rpm for 2 min to precipitate, this cells were resuspended in RPMI 1640 supplemented with 10% FBS to make 5.0×10^5 cells/ml, then irradiated by 3,000 rd of γ -ray.

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T lymphocytes were isolated from blood of healthy adult using Ficoll-hypaque (Amersham, USA), then incubated RPMI 1640 supplemented with 10% FBS to 2.0X10⁶ cells/ml.

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To perform primary Mixed Lymphocyte Reaction (MLR), each 15ml of WT100B1S and T lymphocyte were mixed in 150mm cell culture dish, and incubated for 3 days, then added by 15ml of RPMI 1640 supplemented with 10% FBS and incubated for 3 days further. After incubated for total 6 days, live T lymphocytes were purified using Ficoll-hypaque (Amersham, USA) as described above, and purified T lymphocytes were stored in liquid nitrogen after freezing it by using the media comprising 45% FBS, 45% RPMI 1640, and 10% DMSO.

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After T lymphocytes which were reacted by primary MLR were thawed to perform secondary MLR, the cells were washed with RPMI 1640 media for 2 times and made to be 3.0X10⁵ cells/ml in RPMI 1640 supplemented with 10% FBS.

WT100B1S using as antigen presenting cell was newly cultured by the method as described above, then prepared by irradiation of 3,000 rd of γ-ray and to be 7.5X10⁴ cells/ml in RPMI 1640 supplemented with 10% FBS. After 100μl of prepared WT100B1S was added in 96-well flat bottom cell culture plate and mixed with CD2/Fc and CTLA4/Fc fusion protein at final concentration of 10, 1, 10⁻¹, 10⁻², 10⁻³, and 10^{-4μ}g/ml, 100μl of primary MLR reacted T lymphocytes as above was added. After incubated for 2 days in 5% CO₂, 37°C incubator, 100μl of RPMI 1640 supplemented with 10% FBS was added and incubated for 2 days further. In the last 6 hrs of the total 6 days culture, cells were incubated with addition of 1.2μCi/ml of ³H-thymidine (Amersham, USA).

At the end of culturing, supernatants were removed after centrifugation of 96-well plate was performed at 4°C, 110Xg for 10 min to precipitate T lymphocytes, and pellets were washed with 200µl of 1XPBS. Centrifugation was performed in the same condition and PBS was removed, then 200µl of ice-cold trichloridic acid (TCA, Merck, USA) was added and mixed for 2 min, then reacted at 4°C for 5 min to remove residue of ³H-thymidine.

After centrifugation in the same condition as described above, supernatants were removed and T lymphocytes were fixed by incubation at 4°C for 5 min after 200µl of ice-cold 70% ethanol was added. Supernatants were removed after centrifugation, and ³H-thymidine (Amersham, USA) residue was completely removed by treatment of 10% TCA in the same method as described above.

Cell lysis was performed by reaction with 100µl of 2% SDS (pH 8.0) and 0.5N of NaOH at 37°C for 30min, and T lymphocytes were precipitated by centrifugation at 25°C, 110Xg for 10min, and then 50µl of supernatants was transferred to 96-well sample plate (Wallac, USA). After 1.5 volume of OptiPhase SuperMix (Wallac, USA) was added into the supernatants and mixed for 5 min, the existence of T lymphocyte proliferation was confirmed by measurement of cpm value of ³H using 1450 MicroBeta TriLux microplate liquid scintillation and luminescence counter (Wallac, USA).

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Example 8

Experiment of effect on increase of plasma half-life of glycosylated concatameric fusion dimeric proteins in mouse

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The measurement of plasma half-life of glycosylated concatameric fusion dimeric proteins, [mgTNFR1-TNFR1/Fc]2, [mgTNFR2-TNFR2/Fc]2, [mgCD2-CD2/Fc]2, and [mgCTLA4-CTLA4/Fc]2 was performed by measuring the concentration of proteins using ELISA after 5µg of purified fusion proteins was i.p. injected into mouse (ICR, Samtako, Korea) and bloods were extracted at regular interval for 120 hrs (5 days) as maximum. As shown Fig. 20, Fig. 21, and Fig 22, it could be seen that the plasma half-life of glycosylated concatameric fusion dimeric proteins have been increased in comparison of the corresponding simple fusion dimeric proteins of native shape, and the increase in efficacy through continuous effect could be expected.

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Example 9

Experiment of effects of simple/concatameric TNFR/Fc fusion protein dimers on collagen-induced arthritis of DBA/1 mouse

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Collagen Induced Arthritis (CIA) was developed by injection with 100µg per DBA/1 mouse of type II collagen dissolved at 2mg/ml concentration in 0.05M acetic acid and Arthrogen-CIA adjuvant (Chondrex, USA) into tail. Boosting was performed after 3 weeks, and incomplete Freund's adjuvant (Difco, USA) was used.

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Arthritis was developed 3~4 weeks after immunization with 100µg of type II collagen in the DBA/1 mice. Red and swollen paws of mice had been observed 3~5 days after onset, and inflammatory arthritis lasted more than 3 - 4 weeks. Although inflammation was eventually alleviated, damaged joints remained rigid permanently. The degree of

arthritis was measured 2~3 times per week on the basis of table 12 which represented subjective index of arthritis severity (measure average of five mice in each experiment). To measure the effects of simple and concatameric fusion dimeric TNFR/Fc on CIA, TNFR/Fc or PBS was i.p. injected into the mice. TNFR/Fc was injected with 10µg at every 2 days for 19~45 days into 5 mice per experiments (arrows in Fig. 23). PBS was injected into 5 mice as control. As shown in Fig. 7, in the case of mice injected with existing simple dimeric shaped TNFR/Fc fusion protein, it could be seen that the effect decreased to about 26-38% in comparison with the figures of arthritis index in mice injected with PBS as control, but 42-55% decreased in case of concatameric shaped dimer, [TNFR1-TNFR1/Fc]₂ and [TNFR2-TNFR2/Fc]₂ were injected. Therefore, it could be shown that concatameric fusion dimeric TNFR/Fc fusion proteins have remarkably decreased arthritis of mouse than existing simple fusion dimeric TNFR/Fc fusion proteins.

Table 12. Severity score of arthritis

Severity score	Condition of disease
0	No erythema and swelling
1	Erythema and mild swelling limited to ankle and tarsal
2	Erythema and mild swelling spread from ankle to tarsal
3	Erythema and mild swelling spread from ankle to metatarsal joint
4	Erythema and severe swelling expend to ankle, legs, and digits

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The results as above represented that concatameric shaped dimeric TNFR/Fc fusion proteins were more effective in decreasing the rate of CIA development than existing simple dimeric fusion proteins, therefore, as use in arthritis therapy, concatameric shaped protein compositions could be more effective therapeutics than existing protein compositions.

The concatameric proteins, concatameric fusion dimeric proteins and their glycosylated proteins of the present invention were able to express increased efficacy and high stability, and to be produced with high yield.

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INDUSTRIAL APPLICABILITY

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Address of depositary institution(including postal code and			
Cancer Research Institute, Seoul National University Colle	ge of Medicine		
28 Yongon-dong, Chongno-gu SEOUL 120-091 Republic of Korea			
Date of deposit	Accession Number		
29/06/2001	KCLRF-BP-00046		
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Korea Cell Line Research Foundation(KCLRF) Address of depositary institution(including postal code and c	country)		
Cancer Research Institute, Seoul National University College	ge of Medicine		
28 Yongon-dong, Chongno-gu SEOUL 120-091 Republic of Korea			
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Date of deposit	Accession Number
11/07/2002	KCCM 10399
C.ADDITIONAL INDICATIONS (exceptionkifus applicable)	This information is continued on an additional sheet 🔲
D.DESIGNATED STATES FOR WHICH INDICATION E.SEPARATE FURNISHING OF INDICATIONS (leave to the indications listed below will be submitted to the indications e.q., "Accession Number of Deposit")	
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5 Form PCT/RO/134(July 1998)

0204020244 L ~

DIRECTOR AND

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT R	Rule 13bis)	
A. The indications made below relate to the deposited mic description on page 29, line 51-20	roorganism or other biological material referred to in the	
B. IDENTIFICATION OF DEPOSIT Further deposits are on an additional sheet□		
Name of depositary institution		
Korean Culture Center of Microorganisms(KCCM)		
Address of depositary institution(including postal code and c	ountry)	
361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu, SEOUL 120-091, Republic of Korea	·	
Date of deposit	Accession Number	
11/0//2002	KCCM 10401	
C.ADDITIONAL INDICATIONS (tease blank if not applicable) This	s information is continued on an additional sheet 🗌	
D.DESIGNATED STATES FOR WHICH INDICATIONS A E.SEPARATE FURNISHING OF INDICATIONS (leave black) The indications listed below will be submitted to the In-		
indications e.q., "Accession Number of Deposit")		
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INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCI Rule (30is)		
A. The indications made below relate to the deposited m description on page 27, line 10-20	icroorganism or other biological material referred to in the	
B. IDENTIFICATION OF DEPOSIT Fur	ther deposits are on an additional sheet□	
Name of depositary institution		
Korean Culture Center of Microorganisms(KCCM)		
Address of depositary institution(including postal code and	country)	
361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu, SEOUL 120-091, Republic of Korea		
Date of deposit	Accession Number	
11/07/2002	KCCM 10400	
C.ADDITIONAL INDICATIONS (terreblanki froct applicable) Th	us information is continued on an additional sheet 🗌	
D.DESIGNATED STATES FOR WHICH INDICATIONS	ARE MADE (if the indications are not for all designated States)	
E.SEPARATE FURNISHING OF INDICATIONS(leave b	lank if not applicable)	
The indications listed below will be submitted to the indications e.q., "Accession Number of Deposit")	International Bureau later(specify the general nature of the	
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DELEGOCID AND DELEGOCAT I -

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)			
A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 27, line 10-20			
B. IDENTIFICATION OF DEPOSIT Fur	ther deposits are on an additional sheet 🗌		
Name of depositary institution			
Korean Culture Center of Microorganisms(KCCM)			
Address of depositary institution(including postal code and	country)		
361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu, SEOUL 120-091, Republic of Korca			
Date of deposit	Accession Number		
11/07/2002	KCCM 10402		
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D.DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States) E.SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.q., "Accession Number of Deposit")			
For receiving Office use only	For international Bureau use only		
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apparation			
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INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)		
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Korean Culture Center of Microorganisms(KCCM)		
Address of depositary institution(including postal code and	country)	
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Date of deposit	Accession Number	
11/07/2002	KCCM 10404	
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D.DESIGNATED STATES FOR WHICH INDICATIONS E.SEPARATE FURNISHING OF INDICATIONS (leave b)		
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application	on:	
Authorized officer	Authorized officer	

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)		
A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 29, line 15-20		
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Address of depositary institution(including postal code and country)		
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Date of deposit	Accession Number	
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D.DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)		
E.SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)		
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INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)		
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Address of depositary institution(including postal code and country)		
361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu, SEOUL 120-091, Republic of Korea		
Date of deposit	Accession Number	
11/07/2002	KCCM 10405	
C.ADDITIONAL INDICATIONS (teneblarkij not applicable) This information is continued on an additional sheet		
D.DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)		
E.SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)		
The indications listed below will be submitted to the International Bureau later(specify the general nature of the		
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5 Form PCT/RO/134(July 1998)

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WHAT IS CLAIMED IS:

1. A concatameric protein comprising two soluble domains, in which a N-terminus of a soluble domain of a biologically active protein is linked to C-terminus of an identical soluble domain or a different soluble domain of a biologically active protein.

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2. A concatameric fusion dimeric protein comprising two monomeric proteins formed by linkage of a concatamer of two identical soluble extracellular domains of proteins involving immune response to a hinge region of an Fc fragment of an immunoglobulin molecule, wherein said monomeric proteins are linked by intermolecular disulfide bonds at the hinge region, and having improved stability and therapeutic effects.

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3. The concatameric fusion dimeric protein as set forth in claim 2, wherein the immunoglobulin molecule is IgG.

4. The concatameric fusion dimeric protein as set forth in claim 2, wherein the protein involving immune response is selected from the group consisting of cytokines, cytokine receptors, adhesion molecules, tumor necrosis factor receptors, receptor tyrosine kinases, chemokine receptors and other cell surface proteins which contain a soluble extracellular domain.

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5. The concatameric fusion dimeric protein as set forth in claim 4, wherein the protein is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-17, TNF, TGF, IFN, GM-CSF, G-CSF, EPO, TPO, M-CSF, GHR, IL-13R, IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-9R, IL-15R, TNFR, TGFR, IFNR, interferon-α R, -β R and -γ R, GM-CSFR, G-CSFR, EPOR, cMpl, gp130, Fas (Apo 1), CCR1, CXCR1-4, TrkA, TrkB, TrkC, Htk, REK7, Rse/Tyro-3, hepatocyte growth factor R, platelet-derived growth factor R, Flt-1, CD2, CD4, CD5, CD6, CD22, CD27, CD28, CD30, CD31, CD40, CD44, CD100, CD137, CD150, LAG-3, B7, B61, β-neurexin, CTLA-4, ICOS, ICAM-1, complement R-2 (CD21), IgER, lysosomal membrane gp-1, α2-

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microglobulin receptor-related proteins, and sodium-releasing peptide R.

6. The concatameric fusion dimeric protein as set forth in claim 2, wherein the monomeric protein contains an amino acid sequence of SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 18, or SEQ ID NO: 20.

- 7. A DNA construct encoding a monomeric protein formed by linkage of a concatamer of two identical soluble extracellular domains of a protein involving immune response to a hinge region of an Fc fragment of an immunoglobulin molecule.
- 8. The DNA construct as set forth in claim 7, wherein the immunoglobulin molecule is IgG.
- 9. The DNA construct as set forth in claim 7, wherein the protein involving immune response is selected from the group consisting of cytokines, cytokine receptors, adhesion molecules, tumor necrosis factor receptors, receptor tyrosine kinases, chemokine receptors and other cell surface proteins which contain a soluble extracellular domain.
 - 10. The DNA construct as set forth in claim 9, wherein the protein is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-17, TNF, TGF, IFN, GM-CSF, G-CSF, EPO, TPO, M-CSF, GHR, IL-13R, IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-9R, IL-15R, TNFR, TGFR, IFNR, interferon-α R, -β R and -γ R, GM-CSFR, G-CSFR, EPOR, cMpl, gp130, Fas (Apo 1), CCR1, CXCR1-4, TrkA, TrkB, TrkC, Htk, REK7, Rse/Tyro-3, hepatocyte growth factor R, platelet-derived growth factor R, Flt-1, CD2, CD4, CD5, CD6, CD22, CD27, CD28, CD30, CD31, CD40, CD44, CD100, CD137, CD150, LAG-3, B7, B61, β-neurexin, CTLA-4, ICOS, ICAM-1, complement R-2 (CD21), IgER, lysosomal membrane gp-1, α2-microglobulin receptor-related proteins, and sodium-releasing peptide R.
 - The DNA construct as set forth in claim 7, wherein the DNA construct contains a nucleotide sequence of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 17, or SEQ ID NO: 19.

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12. A recombinant expression plasmid comprising the DNA construct of claim 7 operably linked thereto.

- The recombinant expression plasmid as set forth in claim 12, wherein the recombinant expression plasmid is a pTR11-Top10' plasmid (accession No.: KCCM 10288), a pTR22-Top10' plasmid (accession No.: KCCM 10289), a pCD22Ig plasmid (accession No.: KCCM 10402), or a pCT44Ig plasmid (accession No.: KCCM 10400).
- 14. A host cell transformed or transfected with the recombinant expression plasmid of claim 12.
- The host cell as set forth in claim 14, wherein the host cell is a mammalian cell.
 - 16. The host cell as set forth in claim 14 or 15, wherein the recombinant expression plasmid is a pTR11-Top10' plasmid (accession No.: KCCM 10288), a pTR22-Top10' plasmid (accession No.: KCCM 10289), a pCD22Ig plasmid (accession No.: KCCM 10402), or a pCT44Ig plasmid (accession No.: KCCM 10400).
- 15 17. The host cell as set forth in claim 16, wherein the host cell is a TR11Ig-CHO cell line (accession No.: KCLRF-BP-00046) or a TR22Ig-CHO cell line (accession No.: KCLRF-BP-00049).
 - A method of preparing a concatameric fusion dimeric protein in which disulfide bonds are formed between the hinge regions of two monomeric proteins, comprising the steps of:
 - culturing the transformed or transfected host cell of claim 14 under conditions suitable for expression of a DNA construct encoding a concatameric fusion monomeric protein in which a concatamer of two identical soluble extracellular domains of

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proteins involving immune response is linked to a hinge region of an Fc fragment of an immunoglobulin molecule; and

isolating and purifying a dimeric protein formed by dimerization of the produced monomeric proteins from culture medium.

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- a concatameric fusion monomeric protein is prepared by preparing a DNA construct encoding a simple fusion monomeric protein formed by joining a DNA fragment encoding an Fc fragment of an immunoglobulin molecule and a DNA fragment encoding a soluble extracellular domain of a protein involving immune response; and joining the prepared DNA construct and a second DNA fragment identical to the DNA fragment encoding a soluble extracellular domain of a protein involving immune response.
- 20. The method as set forth in claim 19, wherein the DNA construct encoding a concatameric fusion monomeric protein contains a glycosylation motif sequence.
- The method as set forth in claim 20, wherein the glycosylation motif sequence is inserted to a region at which two soluble extracellular domains are joined.
 - 22. The method as set forth in claim 19, wherein the concatameric fusion monomeric protein contains a leader sequence.
 - 23. The method as set forth in claim 22, wherein the concatameric fusion monomeric protein is CTLA-4, and the leader sequence has an amino acid sequence of MACLGFQRHKAQKNLAARTWPCTLLFFIPVFCKA.
 - 24. The method as set forth in claim 23, wherein the leader sequence has an amino acid sequence of MRTWPCTLLFFIPVFCKA excluding ACLGFQRHKAQKNLAA.
 - 25. The method as set forth in any of claims 18 to 24, wherein the host cell is a mammalian cell.

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26. A concatameric fusion dimeric protein comprising two monomeric proteins formed by linkage of a concatamer of two identical soluble extracellular domains of proteins involving immune response to the hinge region of Fc fragment of an immunoglobulin molecule, wherein said monomeric proteins are linked by formation of intermolecular disulfide bonds at the hinge region and glycosylated, and having improved stability and therapeutic effects.

- The concatameric fusion dimeric protein as set forth in claim 26, wherein the monomeric protein contains an amino acid sequence of SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 22, or SEQ ID NO: 24.
- 10 28. A DNA construct encoding a monomeric protein formed by linkage of a concatamer of two identical soluble extracellular domains of proteins involving immune response to a hinge region of an Fc fragment of an immunoglobulin molecule and containing glycosylation motif peptides.
- The DNA construct as set forth in claim 28, wherein the DNA construct contains an amino acid sequence of SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 21, or SEQ ID NO: 23.
 - 30. A recombinant expression plasmid operably linked to the DNA construct of claim 28.
- The recombinant expression plasmid as set forth in claim 30, wherein the recombinant expression plasmid is a pTR11Ig-MG plasmid (accession No.: KCCM 10404), a pTR22Ig-MG plasmid (accession No.: KCCM 10401), or a pCT44Ig-MG plasmid (accession No.: KCCM 10399).
 - 32. A host cell transformed or transfected with the recombinant expression plasmid of claim 30.

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33. The host cell as set forth in claim 32, wherein the host cell is a mammalian cell.

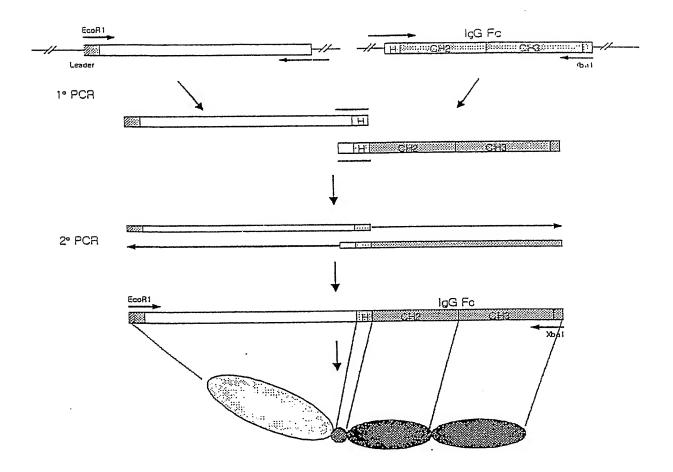
- 34. A pharmaceutical or diagnostic composition comprising the dimeric protein of claim 2.
- 5 35. A pharmaceutical or diagnostic composition comprising the glycosylated dimeric protein of claim 26.

DISCOUNT -1810

PCT/KR02/01427

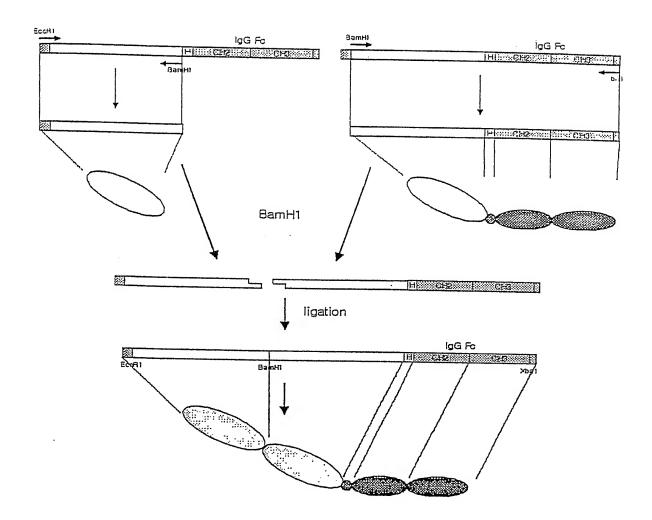
1/23

FIG. 1



2/23

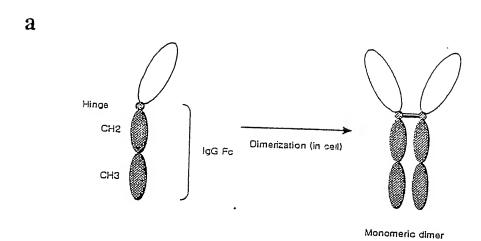
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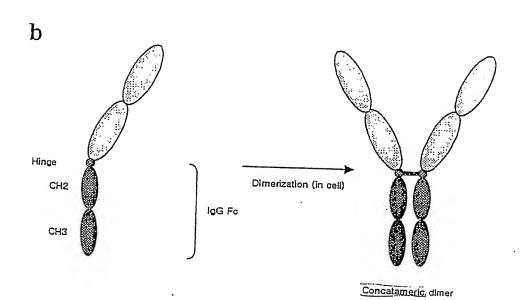


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3/23

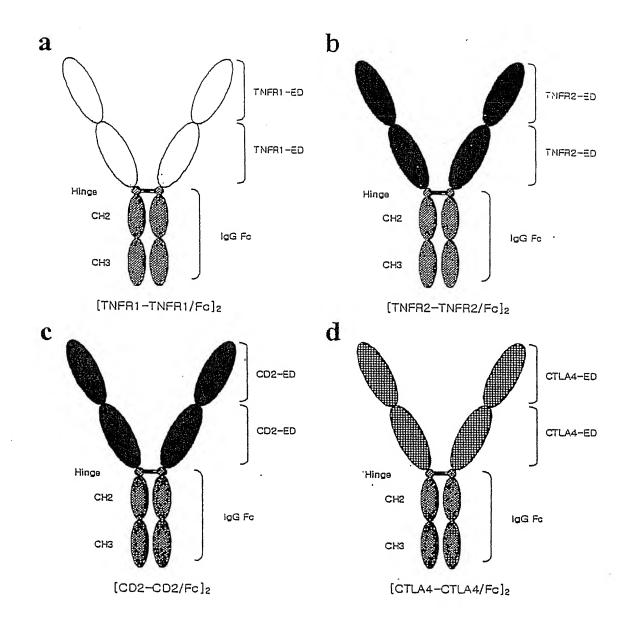
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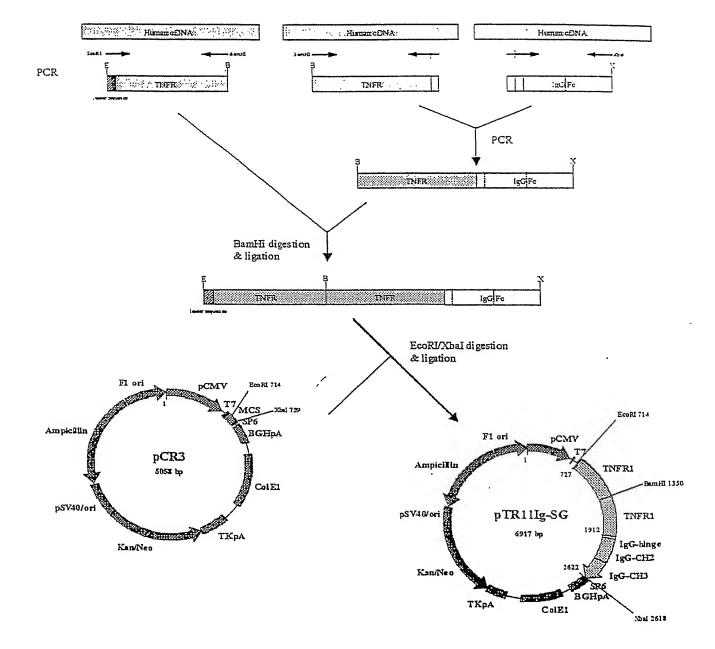
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FIG. 4



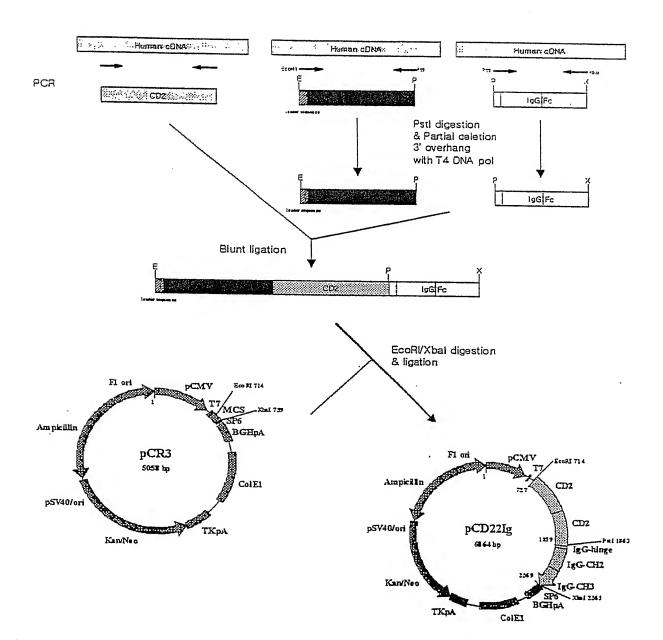
5/23

FIG. 5

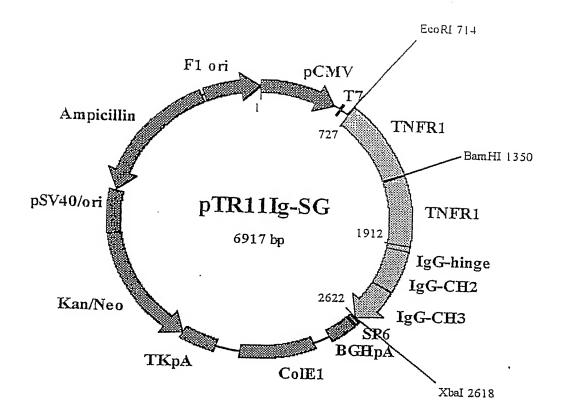


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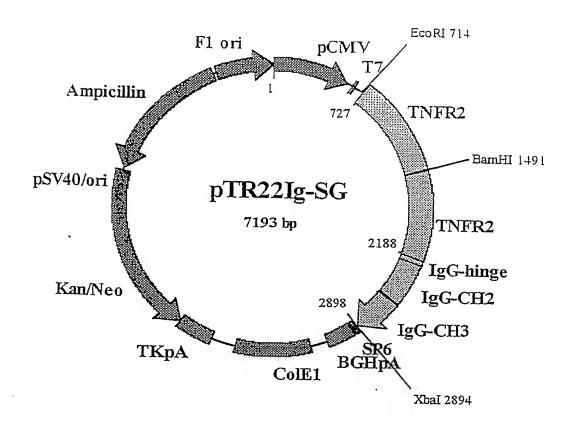
6/23 FIG. 6



7/23 FIG. 7

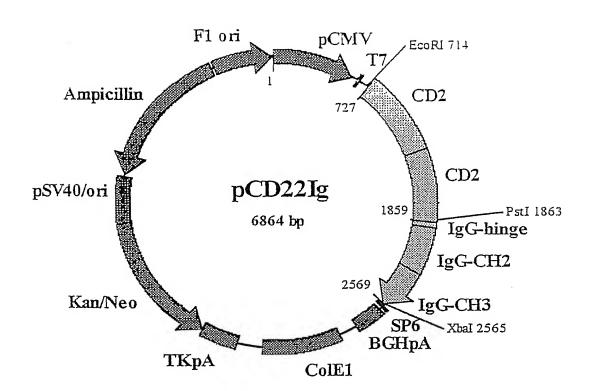


8/23 FIG. 8

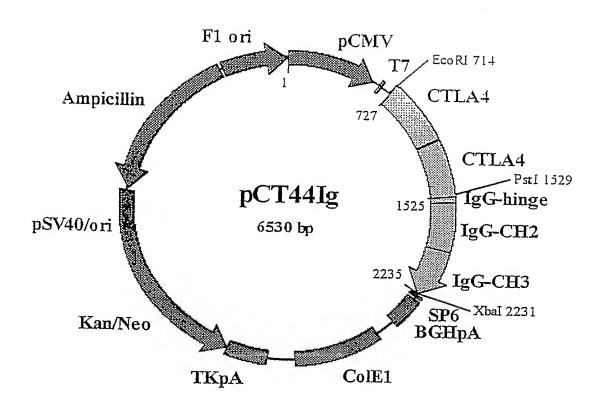


PCT/KR02/01427

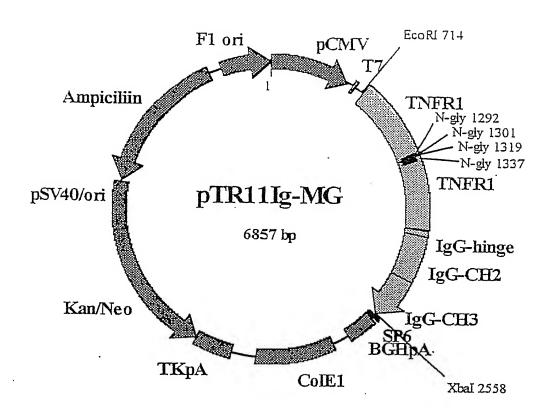
9/23 FIG. 9



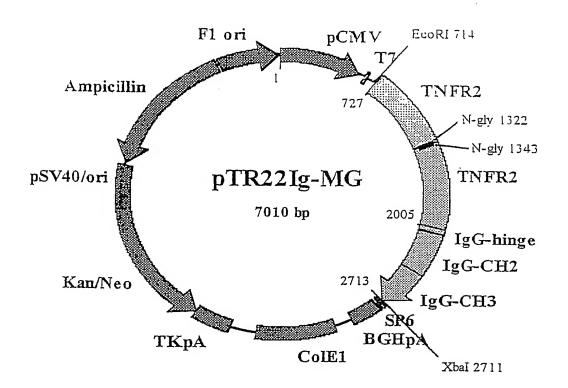
10/23 FIG. 10



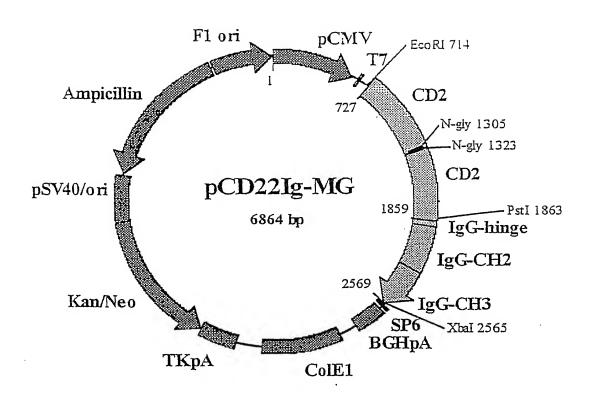
11/23 FIG. 11



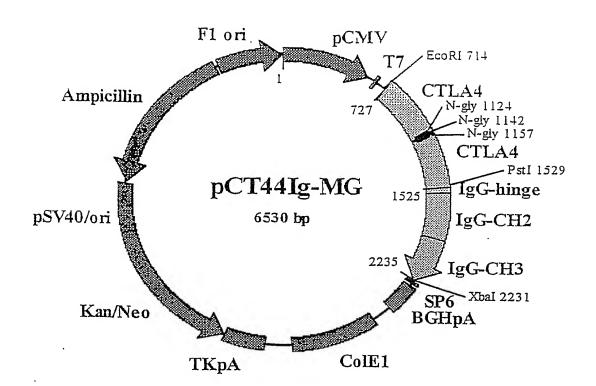
12/23 FIG. 12



13/23 FIG. 13

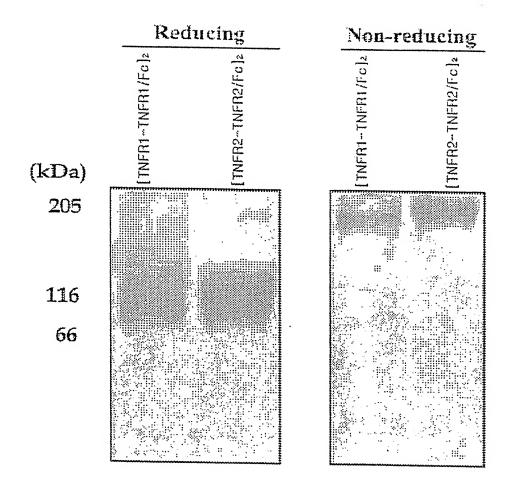


14/23 FIG. 14

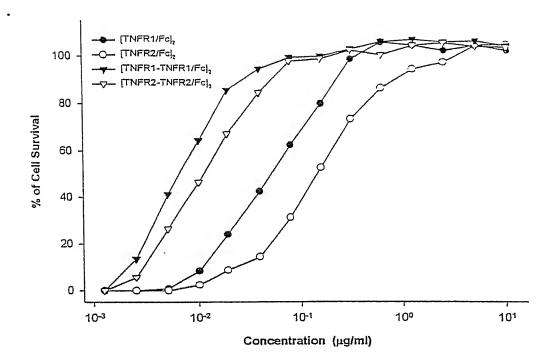


15/23

FIG. 15



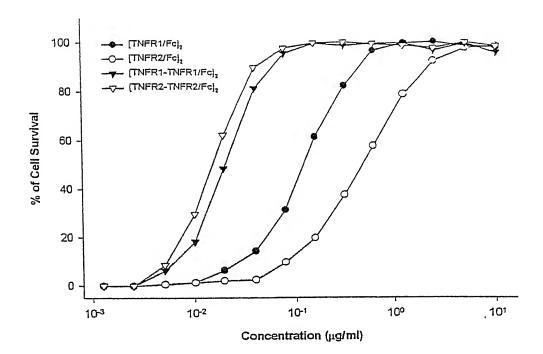
16/23 FIG. 16



PCT/KR02/01427

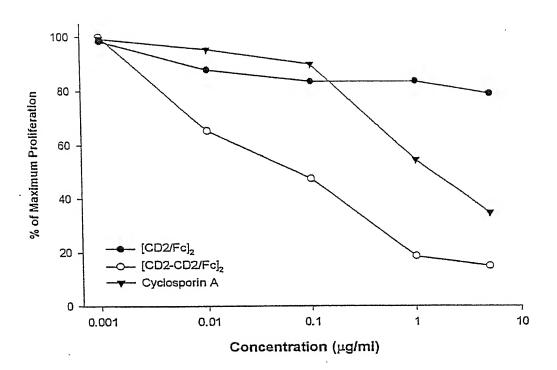
17/23

FIG. 17

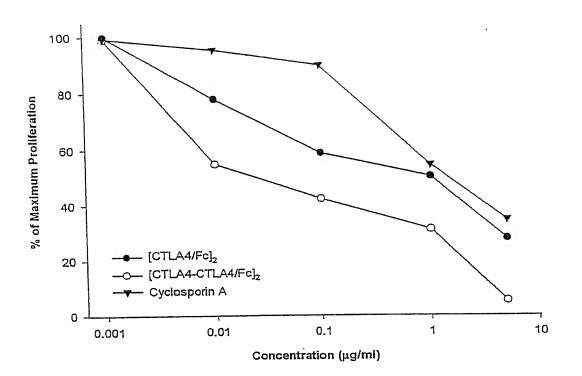


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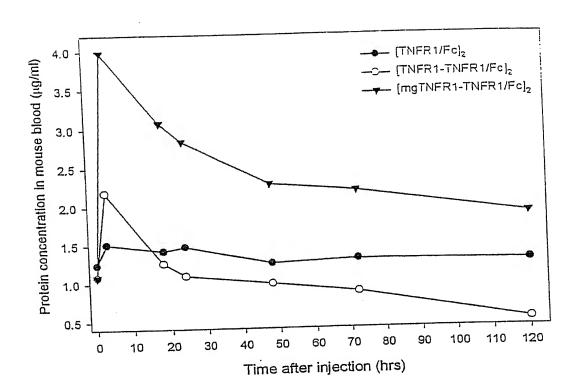
18/23 FIG. 18



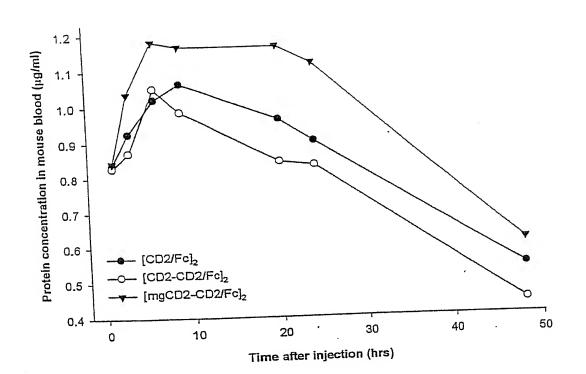
19/23 FIG. 19



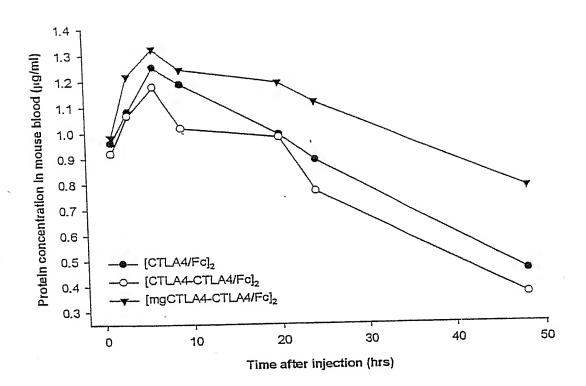
20/23 FIG. 20



21/23 FIG. 21

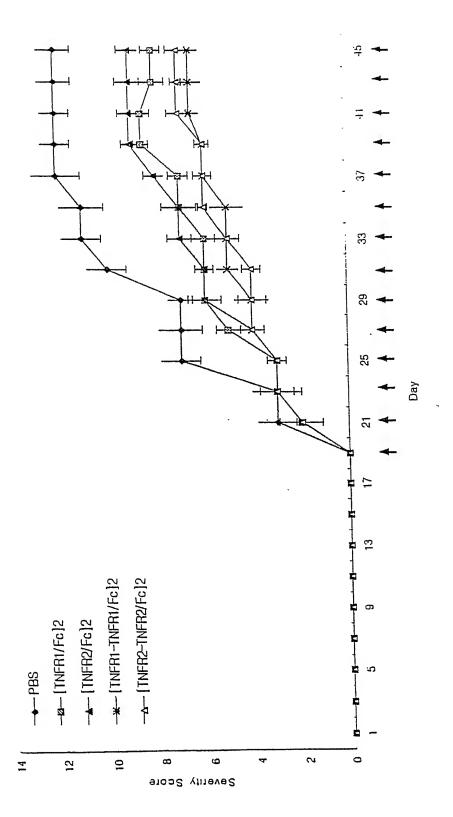


22/23 FIG. 22



23/23

FIG. 23



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          LEE, Hye Ja
          CHOI, Eun Yong
          KIM, Jin Mi
          YIM, Soo Bin
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Gly	Thr	Tyr	ttg Leu	Tvr	Asn	Asn	Cve	Pro	ggc	ccg	ggg	cag	gat	acg	gac	240
65					70	, iop	Oy5	110	G.L.Y	75	GTĀ	GTU	Asp	Thr		
					. •					75		,			80	
tgc	agg	gag	tgt	gag	agc	aac	tcc	ttc	acc	act	tes	~~~	200			
Cys	Arg	Glu	Cys	Glu	Ser	Gly	Ser	Phe	Thr	Ala	Ser	Glu	Aen	uic	CTC	288
				85		_			90			014	71311	95	пец	
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aga	cac	tgc	ctc	agc	tgc	tec	aaa	tgc	cga	aag	gaa	atq	aat	cao	ata	336
Arg	His	Cys	Leu	Ser	Cys	Ser	Lys	Суз	Arg	Lys	Glu	Met	Gly	Gln	Val	550
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Glu	Ile	Ser	Ser	Суѕ	Thr	Val	Asp	Arg	Asp	Thr	Val	Суз	Gly	Cys	Arg	
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aag	aac	cag	tac -	cgg	cat	tat	tgg	agt	gaa	aac	ctt	ttc	cag	tgc	ttc	432
ьуѕ	Asn	Gln	Tyr	Arg	His	Tyr	Trp	Ser	Glu	Asn	Leu	Phe	Gln	Cys	Phe	
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a = +	tas	5.5-	_4-	. .												
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Asn 145	Cys	Ser	Leu	Cys	Leu 150	Asn	Gly	Thr	Val	His 155	Leu	Ser	Сув	Gln	Glu 160	
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3				165	J		-		170	_				175		
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Asn	Glu	Cys		Ser	Суз	Ser	Asn	-	Lys	Lys	Ser	Leu		Суѕ	Thr	
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Lys	Leu	Cys	Leu	Pro	Gln	Ile	Glu	Asn	Val	Lys	Gly	Thr	Glu	Asp	Ser	
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GTÀ	210		ALA	GIU	ELO	дуз 215	THET	Cys	voř	, пуз	220		1111	Cys		•
ccg	tgc	cca	gca	cct	gaa	ctc	ctg	ggg	gga	a ccg	tca	gto	ttc	cto	ttc:	720
Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gl3	/ Pro	Ser	. Val	Phe	Lev	ı Phe	
225	,				230					235	,				240	
				. 220		200	ata	a+a	2+0	. +~~			cct		g gtc	: 768
				_	-			_							ı Val	
				245	_				250			,		25		
aca	a tgo	gt	j gto	ggto	gad	gtg	ago	cac	ga	a gad	e eet	gaq	ggto	aa:	g ttc	816
Th	с Суз	va.			. Asp	v Val	Ser	His	Gl	u Ası	o Pro	o Gli		_	s Phe	3
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aa	e tg	g ta	c gto	g gad	c ggd	e gto	gaç	gto	, ca	t aa	t gc	c aa	g aca	a aa	g ccç	g 864
As															s Pro	
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-	-		-	-		-	•		_			_	-		c acc	
Ar	_		u Gl	n Ty:	r Ası			г Ту	r Ar	g Va			r Va	T l'e	u Thi	Ė
	29	U				29!	5				30	U				
gt	c ct	g ca	.c ca	g ga	c tg	g ct	g aa	t gg	c aa	ıg ga	g ta	c aa	g tg	c aa	g gt	c 960

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Asp	Glu	Leu 355	Thr	Lys	Asn	Gln	Val 360	Ser	Leu	Thr	Cys	Leu 365	Val	Lys	Gly	
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501		. 50.0		405	_	J	1111	Val	410	-	Ser	1119	11,5	415		
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Gly	Asr	n Val	. Phe 420		Cys	Ser	Val	Met 425		Glu	Ala	Leu	430		. His	
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Cys	Arg	Glu	Суѕ	Glu 85	Ser	Gly	Ser	Phe	Thr 90		Ser	Glu	Asn	His 95	Leu
Arg	His	Cys	Leu 100		Cys	Ser	Lys	Cys 105	Arg	Lys	Glu	Met	Gly 110	Gln	Val
Glu	Ile	Ser		Cys	Thr	Val	. Asp 120		Asp	Thr	· Val	Cys 125		Cys	Arg
Lys	Asn 130		ı Tyr	Arg	His	Туг 135	Trp	Ser	Glu	ı Asn	Leu 140		Gln	. Cys	Phe
Asn		s Sei	r Lei	ı Cys	150		n Gly	Thr	. Val	L His		ı Ser	: Cys	: Gln	Glu 160
Lys	s Glı	n Ası	n Thi	r Val		: Thi	r Cys	His	5 Ala		y Fh∈	e Phe	e Lev	175	
Ası	n Glu	а Су	s Va		r Cys	s Se:	r Asr	189		s Lys	s Sei	r Le	ı Glı 190		5 Thr
Lys	s Le	и Су 19		u Pro	o Gli	n Il	e Gli 200		n Va	l Ly:	s Gl	y Th:		ı Asp	Ser

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Pro 225	Cys	Pro	Ala	Pro	Glu 230	Leu	Leu	Gly	Gly	Pro 235	Ser	Val	Phe	Leu	Fhe 240
Pro	Pro	Lys	Pro	Lys 245	Asp	Thr	Leu	Met	Ile 250	Ser	Arg	Thr	Pro	Glu 255	Val
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                                    10
                                                        15
tgg get geg geg eac gec ttg ecc gec eag gtg gea ttt aca ecc tac
                                                                         96
Trp Ala Ala Ala His Ala Leu Fro Ala Gln Val Ala Phe Thr Pro Tyr
             20
                                25
                                                    30
gee eeg gag eee ggg age aca tge egg ete aga gaa tae tat gae eag
                                                                        144
Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln
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aca gct cag atg tgc tgc agc aaa tgc tcg ccg ggc caa cat gca aaa
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		_	-	agc Ser		_	_		-			-	-			336
Ī.			Arg		-		-						Cys		ctg Leu	384
_	-	Gl.n			-		Leu	-		_	-	Arg	_	_	ege Arg	432
	Gly				-	Arg				-	Thr		-		gtg Val 160	480
tgo	: aaç		_	Ala	ceg	. dāā	_		Ser	aac Asn	acg			Ser	acg Thr	528
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				g gat		-	_		g te					c cgg	ı agt ı Ser	624
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PCT/KR02/01427

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Phe	Leu	Leu	Pro	Met	Gly	Pro	Şer	Pro	Pro	Ala	Glu	Gly	Ser	Thr	Gly	
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gac	gca	gag	CCC	aaa	tct	tgt	gac	aaa	act	cac	aca	tgc	cca	ccg	tgc	816
Asp	Ala	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	
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Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	
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Lys	Pro	ГÀЗ	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	
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Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	
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Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	
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						ggc										1104
His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	
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Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	
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ccc	age	gac	atc	acc	ata	.~ ~ .~	+.70	ana	200	t						1000
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ctc	tac	agc	aaq	ctc	acc	ata	asc	aar	auc	200	taa	929		~~~		1200
		Ser														1392
	450		_			455	•	-		,	460			1		
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	Phe	Ser	Суѕ	Ser		Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	
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cag	aag	agc	ctc	tcc	ctg	tct	ccg	ggt	aaa		tqa					1473
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Leu	ı Thi	r Lys	s Ası	Glr 405		. Ser	Leu	Thr	Cys 410		ı Val	. Lys	Gly	Phe 415	-
Pro	Sei	c As _l	2 Ile 420		a Val	. Glu	ı Trp	Glu 425		Ası	ı Gly	Gln Gln	Pro 430		Asn
Ası	туг	r Ly:	s Th	r Thi	r Pro	Pro	Val	. Let	ı Asp	o Sei	r Asp	o Gly	ser Ser	Ser	Phe

BRIGDOOD SMID DROTODOAT I -

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N-linked glycosylation site

Sequence Listing

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4									F 1	•						
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Cys		ьуѕ	ser	Leu	GLU	,,	Thr	гуs	Leu	Суя		Pro	GIn	TTe	Glu	
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His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	
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тyr	Arc	y Val	. Va.l			Leu	Thr	Val			Gln	Asp	Trp		Asn	
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~~-					. 4n			1.	_							
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Gly 65	Thr	Tyr	Leu	Tyr	Asn 70	Asp	Суз	Pro	Gly	Pro 75	Gly	Gln	Asp	Thr	Asp 80
Суз	Arg	Glu	Cys	Glu 85	Ser	Gly	Ser	Phe	Thr 90	Ala	Ser	Glu	Asn	His 95	Leu
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Glu	Ile	Ser 115	Ser	Cys	Thr	Val	Asp 120	Arg	Asp	Thr	Val	Cys 125	Gly	Сув	Arg
Lys	Asn 130	Gln	Tyr	Arg	His	Tyr 135	Trp	Ser	Glu	Asn	Leu 140	Phe	Gln	Cys	Phe
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Lys	Gln	Asn	Thr	Val 165	Cys	Thr	Cys	His	Ala 170	Gly	Ph∈	Phe	Leu	Arg 175	Glu
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Lys	Leu	Cys - 195	Leu	Pro	Gln	Ile	Glu 200	Asn	Val	Lys	Gly	Thr 205	Glu	Asp	Gly

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Glu	Trp	Glu	Ser	Asn 565	_	Gln	Pro	Glu	Asn 570		Туг	Lys	Thr	Thr 575	
Pro	Val	. Leu	Asp 580		Asp	Gly	Ser	Ser 585		. Leu	ı Tyr	Ser	Lys 590		. Thr
Val	. Asp	595	Ser	: Arg	Trp	Gln	Gln 600		/ Asn	ı Val	. Phe	Ser 605		Ser	Val
Met	His		ı Ala	. Lev	ı His	Asn 615		. Tyr	Thr	r Glr	1 Lys		. Leu	ı Ser	: Leu
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	e ege tg r Arg Cy 10	s Ser Se							336

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		115				3		120	Ī			_		125							
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Ser	Lys	Gl	n G	Slu	Gly	Cys	Arg	Leu	Cys	Al	a P	ro	Leu	Arg	Lу	s C	ys	Arg			
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																		~+~		,	480
						gcc														•	400
	GL	r Ph	.e (	эту	Val	Ala 150	Arg	F,LO	GT?	, 111		155	1111	Ser	, T.S	b A	aı	160			
145						130															
tac	aao	a ec	iG.	tat	qcc	ccg	ggg	acg	tto	e to	:C 8	aac	acg	act	to	a t	cc	acg			528
						Pro															
-					165					17							175				
-																		ggg			576
Asp	ıl.	e C	ys	Arg	Pro	His	: Glr	ı Ile	: Су	s As	sn	Val	Val	. Ala			Pro	Gly			
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					,		4-			4.		500	tor		<b>~</b> = 4	~~	raa	ant			624
																		agt Ser			
ASI	n A.		er 95	Mec	YSI	) AT	ı va.	1 Cy 20			CL	****		20			5				
			-						-												
at	g go	c c	ca	ggg	gc:	a gt:	a ca	c tt	a cc	c c	ag	сса	. gt	g tc	c a	ca	cga	tcc	:		672
Me	t A	la P	ro	Gl?	Al:	a Va	l Hi	s Le	u Pr	:o G	ln	Pro	Va	l Se	r T	hr	Arç	g Ser	:		
	2	LO					21	5					22	0							
					-																700
																		to:			720
		is 1	ľhr	Gli	n Pr			:0 G1	.u P	ro S	Ser			a Pr	TO 2	er	Th	r Se:			
22	:5					23	U					235	5					24	O		
<b>t-</b> t	-c c	ta (	ato		a at	a ac	rc ce	ec ac	rc c	cc (	cca	act	t qe	a q	id s	agc	gg	a tc	С		768
		-																y Se			
					24		•				250						25				
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a	ac g	ca	act	ac	a co	ec ta	ac g	cc c	cg g	ag	ccc	gg	g a	gc a	ca '	tgc	cg	g ct	С		816
A	sn A	lla	Th	r Th	r P	ro T	yr A	la P	ro G	lu	Pro	Gl	y S	er T	hr	Cys	Ar	g Le	u		
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Pro		Gln	His	Ala	Lys		Phe	Суѕ	Thr	Lys		Ser	Asp	Thr	Val	
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tat	αac	tcc	tat	σασ	аас	agc	aca	tac	acc	cad	ctc	taa	aac	taa	att	960
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305	-		_		310					315		<b>r</b> -			320	
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Pro	Glu	Cys	Leu	Ser	Cys	Gly	Ser	Arg	Cys	Ser	Ser	Asp	Gln	Val	Glu	
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Thr	Gln	Ala		Thr	Arg	Glu	Gln		Arg	Ile	Cys	Thr	Cys	Arg	Pro	
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Thr	Ser	Pro	Thr	Arg	Ser	Met	Ala	Pro	Gly	Ala	Val	His	Leu	Pro	Gln	
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THE	530		val	Thr	Cys	Val 535	LEV	val	Asp	Val			GLU	Asp	Pro	
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Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	
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Ser	. Val	. Leu	Thi	· Val	Leu	His	Gln	Asp	Trp	Let	Asn	Gly	Lys	Glu	Tyr	
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Lys	-	-	-				-			-						
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Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu	Ser	
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Ser	Asp	G12	/ Ser	Ser	Phe	Leu	Туг	Ser	Lys	Leu	Thr	Val	Asp	Lys	Ser	
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agg	tgg	cag	g caç	dda	g aac	gtc	ttc	tca	ı tgo	: tec	gto	g ato	g cat	gaç	gct	2112
Arg	Trp	Glr	ı Gl.r	Gly	/ Asr	Val	. Fh∈	Sei	Cys	Ser	Val	. Met	His	Glu	Ala	
	690	)				695	ò				700	)				
ctg	cad	c aad	c cac	tac	e acq	ı caç	, aaq	g ago	e cto	to to	ct	g to	ccc	g ggt	aaa	2160
Let	His	s Ası	n His	з Туі	r Thi	Glr	ı Lya	s Se	r Lei	ı Sei	: Lei	ı Se:	r Pro	Gly	y Lys	
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Met	Ala 210	Pro	Gly	Ala	Val	His 215	Leu	Pro	Gln	Pro	Val 220	Ser	Thr	Arg	Ser
Gln	His	ጥh r	Gln	Pro	ሞስ ኮ	Pro	Glu	Pro	Ser	ጥh r	Ala	Pro	Ser	Thr	Ser
225	111.5	****	O111	110	230	110	GLU	110	501	235	7124		501	****	240
Phe	Leu	Leu	Pro	Met 245	Gly	Pro	Ser	Pro	Pro 250	Ala	Glu	Gly	Ser	Gly 255	Ser
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Pro	Glu	Cys	. Leu	325		Gly	Ser	Arg	330		Ser	Asp	Gln	. Val 335	Gl.u
Thr	Gln	a Ala	340		Arg	Glu	Gln	Asn 345	-	Ile	Cys	Thr	Суs 350	_	Pro
Gl3	7 Trp	35!	_	s Ala	. Lev	. Ser	360		ı Glu	ı Gly	7 Cys	365		ı Cys	Ala
Pro	37(		g Ly:	s Cys	s Arç	375	_	7 Phe	e Gly	y Val	380		g Pro	o Gly	Thr
Gl:		r Se	r As	p Val	L Va:	_	s Lys	s Pro	o Cys	39!		o Gly	y Thi	r Phe	Ser 400
Ası	n Th:	r Th	r Se	r Sei 40!		r Asj	o Ile	e Cy	s Arg		o His	s Glı	n Ile	∍ Cys 415	s Asr
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His	Thr	Суѕ	Pro 500	Pro	Cys	Pro	Ala	Pro 505	Glu	Leu	Leu	Glγ	Gly 510	Pro	Ser
Val	Phe	Leu 515		Pro	Pro	Lys	Pro 520	Lys	Asp	Thr	Leu	Met 525	Ile	Ser	Arg
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Glu 545		Lys	Phe	Asn	Trp	_	· Val	Asp	Gly	7 Val. 555		Val.	His	Asn	Ala 560
Lys	Thr	Lys	Pro	Arg 565		Glu	ı Gln	Туг	570		Thr	Tyr	Arg	Val 575	Val
Ser	· Val	. Let	Thr 580		. Leu	ı His	Gln	Asp 585		o Leu	ı Asn	Gly	590		Tyr
Lys	в Сув	595		. Ser	: Ası	ı Lys	600		ı Pro	o Ala	a Pro	605		Lys	Thr
Ile	e Sei 61(	_	s Ala	a Lys	s Gly	y Gl:		o Arç	g G.l.ı	ı Pro	620		l Tyr	Thr	Leu
Pro 62!		Se:	r Arq	g Ası	63		ı Thi	t Ly:	s Ası	n Gl.1 63!		L Sei	r Lei	ı Thı	Cys

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gag at	e tet tet tge	c aca gtg gac o	cgg gac acc gtg tgt	ggc tgc agg	384

Glu	Ile	Ser 115	Ser	Суѕ	Thr	Val	Asp 120	Arg	Asp	Thr	Val	Cys 125	Gly	Cys	Arg		
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Lys	Asn	Gln	Tyr	Arg	His	-	Trp	Ser	Glu	Asn		Phe	Gln	Cys	Phe		
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Asn	Cys	Ser	Leu	Cys	Leu	Asn	Gly	Thr	Val	His	Leu	Ser	Cys	Gln	G.lu		
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Lys	Gln	Asn	Thr	Val	Cys	Thr	Суѕ	His	Ala	Gly	Phe	Phe	Leu	Arg	Glu		
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Asn	Glu	Cys	Val	Ser	Cys	Ser	Asn	Cys	Lys	Lys	Ser	Asn	Glu	Thr	Asn		
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Lys	Thr	Cys	Let	His	Asn	Gly	Ser	Arg	Glu	Lys	Asn	Asp	Ser	Val	. Cys		
		195					200	)				205	•				
ccc	caa	r dds	aaa	a tat	atc	cac	cct	caa	aat	aat	teç	, att	tgc:	tgt:	acc		672
Pro	Gln	Gl3	/ Lys	s Tyr	Ile	Hi.s	Pro	Glr	Asr	ı Asn	Ser	: Ile	Cys	Суз	Thr		
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aac	, tgc	cac	c aaa	a gga	a acc	tac	: ttg	g tac	aat	gac	e tgt	cca	a ggc	cci	g ggg		720
Lys	Cys	s His	s Ly	s Gly	/ Thi	ту	Lei	туі	Ası	n Asp	с Суя	s Pro	Gl3	7 Pro	o Gly		
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Glr	n Asp	o Th	r As	р Су:	s Ar	g Gl	з Су	s Gl	ı Se:	r Gl	y Se	r Ph	a Thi	. Al	a Ser		
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Gli	ı Ası	n Hi	s Le	u Ar	g Hi	s Cy	s Le	u Se	r Cy	s Se	г Ьу	в Су	s Ar	д Гу	s Glu		
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Суѕ	_	Cys	Arg	Lys	Asn		Tyr	Arg	His	Tyr		Ser	Glu	Asn	Leu		
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++0	cac	tac	++0	aat	tac	adc	ctc	tac	ctc	aat	aaa	acc	uta	cac	ctc		960
	_	•		Asn	_	-		-									
305		-1-			310			.,		315	-				320		
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Ser	Суз	Gln	Glu	Lys	Gln	Asn	Thr	Val	Cys	Thr	Cys	His	Ala	Gly	Phe		
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Phe	Leu	Arg			Glu	Cys	Val		Cys	Ser	Asn	Cys			Ser		
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cto	. « » «	r toro	י פרר	. 220	tta	tac	cta	ccc	can	att	gac	r aat	att	230	l ddc		1104
_		•	_		-	-			_						Gly		
		355				_	360					365		-			
act	gaç	g gad	e tea	a ggc	acc	аса	gca	gag	ccc	aaa	ı tct	tg:	gac	aaa	act		1152
Thi	Glu	ı Ası	p Se	r Gly	Thr	Thr	Ala	Glu	Pro	Lys	Se i	r Cy	s Asp	b Lys	s Thr		
	370	)				375	٠				380	)					
		_		-	-		-		-						g tca		1200
		r Cy	s Pr	o Pro			) Ala	ı Pro	) G.I.1			n GT	A ст.	y PI	Ser 400		
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at	c tt	c ct	c tt	c cco	2 008	a aas	1 000	c aad	g ga	acc	c ct	c at	g ato	s to	c cgg		1248
															r Arg		
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Th	r Pr	o G1	u Va	l Th	r Cy	s Va	l Va	l Va	l As	p Va	l Se	r Hi	s Gl	u As	p Pro		
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Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	
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Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	
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Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	
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Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	Cys	
		515					520					5Ω5	•			
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Leu		_	Gly	Phe	Tyr			Asp	Ile	Ala	Val	. Glu	Trp	Glu	Ser	
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	_	Glr	Pro	Glu			Tyr	Lys	Thr			Pro	Val	Leu	Asp	
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Ser	. Ast	Gly	/ Ser	: Phe	Phe	. Lei	ı Tyr	Ser	Lys	Leu	Thi	· Val	l Asp	Lys	Ser	
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agg	t tg	g caq	J cad	g ggg	g aad	gto	ttc	tca	a tgo	e tec	gt	y ato	g cat	gag	g gct	1776
Arç	Tr	Glı	n Gli	n Gly	/ Ası	va.	l Phe	Sei	с Суз	s Sei	· Va.	l Me	t His	s Glu	ı Ala	
			580	0				585	5				590	)		
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Phe 309		n Cy	s Ph	e As	n Cy 31		r Le	u Cy	s Le	u As 31		y Th	ır Va	al H:	is	Leu 320
Se	r Cy	s Gl	ln Gl	u Ly 32		n As	n Th	ır Va	.1 C ₃		ır C3	s Hi	is A		1у 35	Phe
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Le	u Gl		ys T) 55	ır Ly	s Le	eu C		∋u Pi 60	co G.	ln I	le G		sn V 65	al I	ys	Gly

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Ьys	s Су.	s Ly	s Va	.l Se	er As 85	n Ly	rs Al	a L		Pro 490	Ala	Pro	) Il	e Gl		ys 195	Thr
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Pr	o Pr		er Ai 15	eg A	sp Gi	Lu Le		hr I 20	ъ'ns	Asn	Glı	n Vai	l Se 52		eu '	ľhr	Cys
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Gln	Met 50	Ser	Asp	Asp	Ile	Asp 55	Asp	Ile	Lys	Trp	Glu 60	Lys	Thr	Ser .	Asp
Lys 65	Lys	Lys	Ile	Ala	Gln 70	Phe	Arg	Lys	Glu	Lys 75	Glu	Thr	Phe	Lys	Glu 80
Lys	Asp	Thr	Tyr	Lys 85	Leu	Phe	Lys	Asn	Gly 90	Thr	Leu	Lys	Ile	Lys 95	His
Leu	Lys	Thr	Asp	Asp	Gln	Asp	Ile	Tyr 105	Lys	Val	Ser	Ile	Tyr 110	Asp	Thr
Lys	Gly	Lys		Val	Leu	Glu	Lys 120		Phe	Asp	Leu	Lys 125		Gln	Glu
Arģ	Val		. Lys	: Pro	Lys	Ile 135	s Ser	Trp	Thr	Cys	11e		Thr	Thr	Leu
Th:		s Glı	ı Val	. Met	: Asn 150		y Thr	: Asp	Pro	Glu 155		Asn	ı Leu	Tyr	Gln 160
Ası	p Gly	y Ly	s Hi	s Let		. Lei	ı Sei	c Glr	170		L Il∈	· Thi	T His	. Lys 175	
Th	r Th	r Se	r Le		r Ala	a Ly	s Ph	≥ Ly:		s Th:	r Ala	a Gl	y Asr 190		val
Se	r Ly	s Gl		r Se	r Va.	l Gl	u Pr 20		l Se	r Cy	s Pr	o Al.		u Pro	b Lys
Se	r Cy 21		эр Ьу	s Th	r Hi		r Cy .5	s Pr	o Pr	о Су	s Pr 22		a Pr	o Glı	ı Leu
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Le	eu M∈	∍t I.	le Se	er Ar 24		ır Pi	ro Gl	lu Va	al Th		/s Va	ıl Va	ıl Va	l As	p Val 5
S	er H	is G	lu A	sp Pi	ro Gl	Lu V	al L	ys Pl	ne As	sn Ti	רף די	/r Va	al As	sp Gl	y Val

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385				1	390	I-	1			395	200	- 1 -	502	2,72	400
Th r	Val	Asn	Lvs	Ser	Arg	ጥተተ	(2) n	(E) n	Glv	- 7) en	Val	Pho	Son	Cue	Sor
****	V GL	1101	כענ	405	71119	111	GLII	0111	410		Val	rne	per	415	Set
Val	Mo t	111.	. C1	71.	7	TT3 _	71	774	m	m1	G1	<b>T</b>		<b>.</b>	
val	. rie L	urs	420		Leu	urs	ASII	425		inr	GTU	глуѕ	430	ren	Ser
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Cys	Lys	Val	Glu	Leu	Met	Tyr	Pro	Pro	Pro	Tyr	Tyr	Leu	Gly	Ile	Gly	
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TAO					100					1.50						
			-+-	ctg	~~~	~~~	000	+	at a	++0	at a	++0	ccc	cca		528
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Pro	ГЛS	.Ası			Met	. ITe	Ser			. Prc	) G-11	r val			· Val	
			180	)				189	)				190			
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Val	. Val	L As	o Vai	l Sei	His	s Glu	ı Ası	) Pro	o Gli	ı Val	LLy			Trp	Tyr	
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gto	gad	g g	c gt	g gaq	ggt	g cat	aa	t gc	c aaq	g aca	a aa	g acc	g cgg	g gaq	g gag	672
Va.	l As	o Gl	у Vа	l Gl	ı Va.	l His	e Ası	n Al	a Ly:	∃ Th:	r Ly	s Pro	o Arç	g Glı	ı Glu	
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ca	g ta	c aa	c ag	c ac	g ta	c cg	j gt	g gt	c ag	c gt	c ct	c ac	c gt	c ct	g cac	720
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са	g ga	c tọ	ıg ct	g aa	t gg	c aa	g ga	g ta	.c aa	g tg	с аз	g gt	c to	c aa	c aaa	768
Gl	n As	p Tr	p Le	u As	n Gl	у Ьу	s Gl	u Ty	r Ly	s Cy	s Ly	rs Va	l Se	r As	n Lys	
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													Phe			
•				325				•	330	•	•			335		
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Phe	Ser	Суз	Ser	Val	Met	His	Glu	Ala	Leu	His	. Asr	n His	Tyr	Thr	Gln	
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aaç	g ago	cto	e tec	e etg	, tct	ccg	ı ggt	aaa	à		to	ја				1134
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Pro	. Lys	s Asp	Thr 180		Met	Ile	Ser	Arg		Pro	Glu	Val	. Thr 190		; Val
Va.	L Val	l Asp 199		l Ser	His	: Glu	. Asp		Glu	ı Val	. Lys	205		Trp	Tyr
Va.	1 As _j		y Va.	l Glı	ı Val	. His		Ala	Lys	5 Thr	Lys 220		Arc	g Glu	ı Glu

Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His 230 235 Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys 250 245 Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln 270 265 260 Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu 275 280 Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro 290 295 300 Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn 315 305 310 Tyr Lys Thr Thr Fro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu 325 330 335 Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val 350 345 340 Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln 365 355 360 Lys Ser Leu Ser Leu Ser Pro Gly Lys 370 375 <210> 17 <211> 1854 <212> DNA <213> Homo sapiens <220> <221> CDS (1)..(1851) <222>

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Val	Ser	Ser	Lys	Gly	Ala	Val	Ser	Lys	Glu	Ile	Thr	Asn	Ala	Leu	Glu	
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Гуs	Lys	Lys	: Ile	Ala	Gln	Phe	Arg	Lys	Glu	Lys	Glu	Thr	Phe	Lys	Glu	
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Lys	Asp	Th:	r Tyr	Lys	Leu	Phe	Lys	Asn	Glz	y Thr	Lev	Lys	: Ile	: Lys	His	
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Lev	Lys	s Th	r Asp	Ası	o Glr	ı Asp	) Ile	туг	Lys	s Val	L Se	c Ile	∍ Туз	r Asj	o Thr	
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Ası	210		ı Glı	u Thi	r Trp	215 215		. Leu	GLY	Gln	220		a Asr	ı re.	ı Asp	
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Lу	s Th	r Se	r As	р Ly 24		s Ly:	s Ile	∍ Ala	a Gli 250		e Ar	g Ly	s Gl	u Ly. 25	s Glu 5	
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Th	r Ph	ie Ly	rs G] 20		s As	p Th	г Ту	r Ly:		u Ph	е Ілу	s As	n Gl 27		r Leu	
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WO 03/010202 PCT/KR02/01427

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Asn	Leu	Tyr	Gln	Asp	Gly	Lys	His	Leu	Lys	Leu	Ser	Gln	Arg	Val	Ile	
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ınr	HIS	. Буs 355	-	The	Inr	per	360		Ald	пуз	rne	. шуз 365	-	1111	Ala	
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Gly	Asr	ı Lys	s Val	Ser	Lys	Glu	Ser	Ser	Val	Gli	Pro	Val	Ser	Cys	Pro	
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Gln	Tyr	Asn	Ser	Thr		Arg	Val	Val.	Ser		Leu	Thr	Val	Суз			
465					470					475					480		
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															aaa Lys		1400
GIN	Asp	Trp	ьeu	485		гус	GTII	тЪт	490	Суз	nys	VGI	Der	495			
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Pro	Arg	g Gli	ı Pro	Gl:	ı Val	. Туг	Thr	Lev	Pro	Pro	Sei	Arç	, Asp	Glu	ı Leu		
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Thi	Ly	s Ası	n Gl	n Va	l Se:	r Lei	ı Thi	Cy:	s Lei	Va.			y Phe	э Ту	r Pro		
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-	-														c aac		1680
		ь тт	e AT	a va	55		. GT.	u se	r Ası	55		n er	0 (37)	u As	n Asn 560		
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t.a	с аа	o ac	c ac	a cc	t cc	c at	a ct	a aa	c to	с па	c aa	c tc	c tt	c tt	c ctc	:	1728
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Pl	ne Se	er C	ys S	er V	al Me	et Hi	.s G]	lu Al	la Le	u H	is As	sn Hi	is Ty	/r Tl	nr Gl	n	
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PCT/KR02/01427

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Thr Cys Glu Val Met Asn Gly Thr Asp Pro Glu Leu Asn Leu Tyr Gln

150

155

145

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Ile	е Ту 29		æp	Thr	. PA:	s G1:	y Ly 29		n Va	l I	eu	Glu	. <b>Б</b> уз		∍ Pho	a As _]	p Leu
<b>L</b> у 30		e G	ln	Glu	ı Ar	g Va 31		r Ly	s Pı	o I	уs	11e		r Tr	p Th	r Cý	s Ile 320
As	n Th	ur T	Phr	Lei	ı Th 32		s Gl	.u Va	ıl Me		Asn 330		y Th	r As	p Pr	o Gl 33	u Leu 5
As	sn Le	∍u '	Tyr	G1:		p Gl	y Ly	/s Hi		eu 45	Lys	Le	u Se	r Gl	Ln Ar 35		al Ile
Tì	nr H	_	-					∍r Lo 3					s Ph		ys Cy 65	ys Th	nr Ala

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Ala	a Lei	u Pr	o Al:		o Ile	e Glu	. Lys	50!		e Sei	. Lys	s Ala	Lys 510		· Gln
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T	r Ly	ys Tì	nr Th	nr Pr 56		o Va	l Le	u As	p Se 57		p Gl	y Se	r Ph	e Ph 57	e Let 5
m.	C	7.	T.	ml	· · · 1/-	1 7\-	. Ta	ra Ca	· ~ 71 ~	-cr Tr	.n G1	n Gl	n Gl	w Ac	n Vai

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++-	_4_					. 4		.~.~					224		a gtg		288
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File	Ter	. vol	i vsř	85		: Суз	1111	GT.7	90		. Der	. Cry	161	95			
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200	· c+	. 20	h atr	י רפי	a orona	e cti	i ann	ז מכי	e atr	ת מים	. acc	ı das	otc	: tar	c atc		336
															r Ile		220
List		~ 111.	100			, 100	(	10	_		- ++41		110				
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-	_				_	tac Tyr		-				_				384
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Gl	n Il	е Ту	r Va. 26		e As	p Pro	o Gli	26		s Pro	o Asj	p Se:	r Ala 270		ı Pro	

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БУЗ	Der	275	ЛЗР	БуЗ	1111	1111	280	0,12			-,-	285					
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ren	љеи 290	атЛ	GTÀ	PLO	per	295	LIIM	пеп	IIIe	TIO	300	цуэ	110	БуЗ	, no p		
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Let	37		у гу	s Gli	л Ту:	r Буя 379		e pā:	s Va.	L Sei	38:		s Al:	a rei	ı Pro		
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G1	n Va	ıl Se	er Le		ır Cy	s Le	u Va	il Ly 42		y Ph	ie Ty	r Pr	0 Se		p Ile	ı	

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Le 46		nr V	al As	sp Ly	rs Se 47		g Tr	p Gl	n Gl	n Gl 47		n Va	l Fh	e Se:	r Cys 480
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Arc			r Ly:	s Pro	b Lys			r Trp	Thr	Суз			n Thi	r Thi	Leu		
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03010202A1 | >

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	1	- 41			5 шу 5	- L11	- va	47mL	a 5e 1		تابد ب			1		
					J				.4.	J				_	~	
Va	l Se	r Se	er Ly	rs Gl	y Al	a Va	l Se	r Ly	s Gl	u Il	e Th	ır As	n Al	a Le	u Glu	

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G	LU L	≠u_M 	et T		15	.U P1	.0 1	λτ τ		50 50	<u>y</u> -	. 4. 5	<b>-</b> + y	,	25		•	

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#### PCT/KR02/01427

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#### INTERNATIONAL SEARCH REPORT

International application No. PCT/KR02/01427

#### CLASSIFICATION OF SUBJECT MATTER A.

IPC7 C07K 16/46

According to International Patent Classification (IPC) or to both national classification and IPC

#### FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C07K 16/46, C07K 19/00, C12N 15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Korean Patents and applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

Medline, Biosis

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X, P  Y,P	EP1148065 A1 (ROSE-JOHN, STEFAN) 24 OCTOBER 2001 see column3, lines 20-40, claims	1  2-5, 7-10, 12, 14, 15
Y	EP0464533 A1 (HOECHST AKTIENGESELLSCHAFT) 8 JANUARY 1992 see claims	2-5, 7-10, 12, 14, 15
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		Further documents are listed in the continuation of Box C.
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See patent family annex. X.

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Date of mailing of the international search report

than the priority date claimed.

Date of the actual completion of the international search

11 DECEMBER 2002 (11.12.2002)

12 DECEMBER 2002 (12.12.2002)

Name and mailing address of the ISA/KR



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Facsimile No. 82-42-472-7140

HAN, Hyun Sook

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Telephone No. 82-42-481-5596



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